ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL HEART AND LUNG INSTITUTE

FISCAL YEAR 1971
PART II

U. S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

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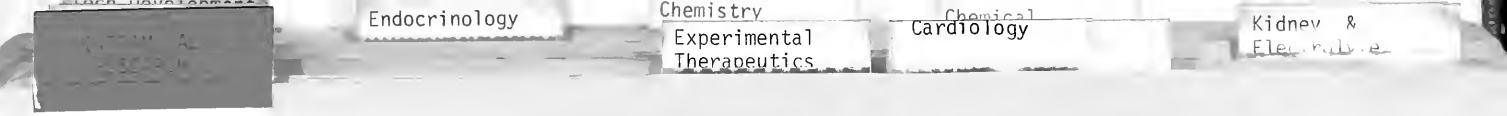
Endocrinology
Chemistry
Experimental
Therapeutics
Cardiology
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INTRAMURAL RESEARCH

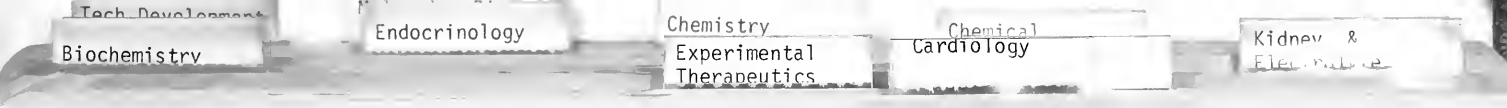
NATIONAL HEART AND LUNG INSTITUTE

July 1, 1970 - June 30, 1971

INTRAMURAL RESEARCH

Project Reports

Cardiology Branch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In the Cardiology Branch two new major areas of research interests have been developed over the past three years. These relate, first, to the investigation of the enzymatic and molecular mechanisms responsible for modulating the contractile state of the myocardium, and second, to the study of myocardial ischemia, myocardial hypoxia, and coronary artery disease. Our investigative efforts in these two areas have been considerably expanded this past year with emphasis on both the physiologic, pharmacologic, and subcellular alterations caused by myocardial ischemia, and on how the ischemia-induced changes can be modified by interventions that may have therapeutic potential.

MOLECULAR MECHANISMS RESPONSIBLE FOR CARDIAC CONTRACTION AND FOR THE PHYSIOLOGICAL EFFECTS OF INOTROPIC AGENTS

Normal Mechanisms

The positive inotropic effects of catecholamines have been postulated to result from increases in intracellular levels of cyclic AMP (C-AMP). Although considerable evidence exists that is compatible with such a concept, it is still uncertain whether stimulation of the adenyl cyclase-C-AMP system and enhancement of contractility are causally related or merely coincidental events. For example, in a recent study we have shown that while 10^{-6}M norepinephrine had a positive inotropic effect within 10 seconds, a significant increase in C-AMP concentration could not be demonstrated until 20 seconds had elapsed. Moreover, preliminary data appear to indicate that lower concentrations of norepinephrine, while still having an appreciably positive inotropic effect, do not alter myocardial C-AMP concentration. Although there are several explanations for this apparent dissociation of the inotropic and enzymatic effects of norepinephrine, these findings do raise the possibility that the norepinephrine-induced stimulation of the adenyl cyclase-C-AMP system is not causally related to the coincidental enhancement of myocardial contractility that occurs.

Two additional studies bearing on this relationship are now in progress. If there is a causal relationship between intracellular C-AMP concentration and contractility, inhibition of phosphodiesterase, the enzyme responsible for inactivating C-AMP, would be expected to enhance the cardiac effects of norepinephrine and glucagon. This was ostensibly verified when we found that while theophylline (a phosphodiesterase inhibitor) did not alter the inotropic effects of calcium (an agent not believed to exert its inotropic effects through the C-AMP system), it caused marked potentiation of the inotropic effects of norepinephrine and glucagon. However, the preliminary results of another investigation have shown that at a concentration of theophylline that increases papillary muscle tension by approximately 50%, no increase in intracellular levels of cyclic AMP occurs. Thus, if the direct inotropic effects

of theophylline are not mediated by the C-AMP system, it is possible that its potentiation of the inotropic effects of norepinephrine and glucagon is also caused by a mechanism independent of C-AMP. To further elucidate this problem, we are in the process of determining whether an increase in intracellular levels of C-AMP occurs when the inotropic effects of norepinephrine are enhanced by theophylline.

Current theory regarding excitation-contraction coupling in cardiac and skeletal muscle centers around the concept that calcium, released by the sarcoplasmic reticulum (SR) following electrical depolarization, diffuses from SR to a receptor located on the actin filament. This event is then believed to eventuate in the interaction of actin and myosin filaments, thereby initiating muscle shortening. Implicit in this concept is the assumption that the rate of diffusion of calcium is sufficient to account for the rapid sequences of contraction and relaxation characteristic of cardiac and skeletal muscle. However, the only data relating to the rate of calcium ion movement in muscle cytoplasm indicate that mobility is at least 50 to 100 times slower than in water, a rate probably too slow to be compatible with the above hypothesis. We recently constructed an analog circuit for the solution of diffusion problems and our results also suggest that the classical hypothesis for excitation-contraction coupling may be in error. Further studies are being performed in skeletal muscle to determine the feasibility of the calcium theory for excitation-contraction coupling.

We have shown previously that ouabain causes negative inotropic responses in cat papillary muscles when external calcium concentration is low. Recent studies have demonstrated that this negative inotropic effect is concentration-dependent and is specific for the digitalis glycoside insofar as norepinephrine, isoproterenol, and angiotensin II produce their usual inotropic responses independent of external calcium concentration. These data could be explained as follows: ouabain results in greater availability of Ca to the contractile apparatus, either by mobilizing a particular intracellular compartment of calcium or by enhancing calcium entry into the cell; this results in enhanced contractility. However, in an external environment of low calcium, either part of the mobilized intracellular calcium moves out of the cell or the net flux into the cell becomes negative, thereby causing a decrease in contractility. These results would seem to be further evidence that the positive inotropic effect of ouabain is related to alteration of intracellular calcium stores.

It has been suggested that one of the actions of digitalis essential for its positive inotropic properties is inhibition of Na^+, K^+-dependent ATPase. It also has been suggested that diphenylhydantoin (DPH) exerts a protective effect on digitalis-induced arrhythmias by interfering with the digitalis-Na^+, K^+-ATPase interaction. If this latter hypothesis were correct, it would imply that the mechanism for the inotropic activity of digitalis is independent of inhibition of Na^+, K^+-ATPase, since DPH does not interfere with the inotropic effects of digitalis. We found, however, that the antiarrhythmic effects of DPH cannot be attributed to prevention of digitalis-induced inhibition of Na^+, K^+-ATPase. Thus, there is still no convincing evidence that seriously questions the concept that the interaction between digitalis and Na^+, K^+-ATPase is basic to the inotropic effects exerted by this agent.

Myocardial Failure

Glucagon exerts a positive inotropic effect in normal hearts, an effect believed to be mediated by C-AMP. However, we previously have shown that its capacity to increase myocardial contractility and to stimulate adenyl cyclase activity are markedly impaired in animals with chronic cardiac failure. To assess directly the influence of chronic heart failure on the effectiveness of glucagon as an inotropic agent in human myocardium, we measured its effect on contractility and adenyl cyclase activity in left ventricular papillary muscles obtained from patients at mitral valve replacement. These studies demonstrated that chronic cardiac failure is uniformly associated with a complete loss of the capacity of glucagon to enhance contractility and stimulate adenyl cyclase, findings that probably explain the disappointing clinical results of this drug in the treatment of patients with prolonged cardiac decompensation.

Myocardial Ischemia and Hypoxia

To determine the mechanisms whereby ischemia results in impaired myocardial function, studies have been initiated to determine the time-dependent sequence of irreversible subcellular changes occurring during ischemia. Preliminary results have demonstrated that subcellular systems differ considerably in their sensitivity to ischemia. For example, Na^+, K^+-ATPase is remarkably stable, whereas mitochondrial preparations show rapid loss of respiratory control and rates of oxygen consumption; adenyl cyclase responsiveness to hormonal stimulation appears to be indetermediate. Parallel studies also are in progress in which ischemic-induced reversible and irreversible changes in myocardial contractility will be correlated with subcellular functional aberrations. After defining some of the subcellular mechanisms responsible for impaired myocardial function during ischemia, we hope to develop techniques whereby ischemia-induced subcellular injury, and therefore physiologic function, can be minimized.

Little information is available concerning the effects of hypoxia or ischemia on the capacity of the heart to respond to various inotropic agents. During the course of studies designed to investigate this problem, we found that in a papillary muscle preparation the positive inotropic actions of two analogues of angiotensin II had unique characteristics, i.e., inotropic potency was enhanced by hypoxia. In contrast, the concentration response curves of both norepinephrine and ouabain were depressed when the papillary muscle was exposed to low oxygen tensions. In order to evaluate the effects of hypoxia on the responsiveness of the <u>intact</u> heart to various inotropic agents, a dog heart-lung preparation was developed in which afterload, preload, and heart rate can be held constant. In this preparation, in contrast to cat papillary muscle, the contractile response to norepinephrine was <u>augmented</u> during hypoxia, the dose-response curve shifting to the left. Furthermore, no deterioration of the preparation occurred after the highest doses of norepinephrine employed. Studies are in progress to evaluate the possible biochemical or physiologic basis of this phenomenon. The effects of hypoxia, as well as ischemia, on the response to other inotropic agents also will be evaluated.

Treatment of Coronary Artery Disease

a) Acute Myocardial Ischemia. Approximately 40% of patients who suffer an acute myocardial infarction die, and two-thirds of these deaths occur within the first few hours, before the patient is admitted to a hospital. Death under such circumstances is probably due to cardiac arrhythmias. To evaluate 1) the type of arrhythmias occurring during the early phases of myocardial infarction and 2) the relative efficacy of various antiarrhythmic agents in this setting, we studied 40 closed-chest conscious dogs in which acute myocardial ischemia was produced by inflating a balloon cuff previously implanted around the left anterior descending coronary artery just distal to its first diagonal branch. About one-half of the dogs did not develop arrhythmias during the first hour of occlusion. In 60 per cent of these, however, sudden release of occlusion was followed 15 seconds to 3 minutes later by ventricular arrhythmias which progressed rapidly to ventricular tachycardia (VT). Reocclusion of the coronary artery abolished the VT with a return to a sinus mechanism in all dogs with release arrhthmias. Increasing the heart rate by either pacing or the administration of atropine prevented the reappearance of VT when the occluding balloon was again released. These results indicate that 1) sudden reperfusion of a previously ischemic region of myocardium, as may occur in man by lysis or dislodgment of a clot, may be responsible for some of the serious arrhythmias seen in acute myocardial infarction which result in sudden death, and 2) increasing the heart rate by the administration of atropine may be successful in the prevention of many, but not all, of these arrhythmias. In contrast to this group, the remainder of the dogs developed frequent and persistent ventricular ectopic beats or episodes of VT within the first one to two hours of occlusion. In most of these dogs increasing the heart rate with atropine or administering lidocaine markedly decreased or completely abolished the arrhythmias. In several dogs, however, the administration of atropine or lidocaine alone was only partially effective, and ectopic activity was eliminated only after administration of the combination of these two drugs.

Although, as indicated above, atropine is effective in treating arrhythmias occurring during the onset of acute myocardial ischemia, it is not known whether the atropine-induced increase in heart rate has any deleterious effect on the degree of myocardial ischemia. We therefore studied the S-T segment response (obtained from 10 to 12 implanted intramyocardial electrodes) to repeated five-minute occlusions of the left anterior descending coronary artery in closed-chest conscious dogs in which we previously had implanted an inflatable balloon around the left anterior descending coronary artery. We found there was a direct linear relationship between heart rate (range 35-150 beats/min) and the degree of S-T segment elevation. We conclude that if the degree of S-T elevation reflects severity or extent of ischemia, increases in rate in experimentally produced acute myocardial ischemia are associated with increases in the degree of myocardial ischemia. Thus, when atropine is administered to control bradycardia-induced arrhythmias during acute myocardial ischemia, the lowest effective dose should be used and excessive increases in rate avoided. There also seems to be no justification for the routine administration of atropine, regardless of rate, to a patient already admitted to a

coronary care unit and under constant observation since arrhythmias can be treated as they occur, thereby avoiding the deleterious effect of unnecessary increases in heart rate on the degree of myocardial injury.

The Montgomery County Heartmobile is a mobile coronary care unit capable of delivering emergency resuscitative treatment to any patient in a defined geographic area within 5 minutes of alert. We are currently engaged in a collaborative effort to determine the type of arrhythmias that occur during the prehospital phase of acute myocardial infarction in man and to determine the relative efficacy of atropine and lidocaine in the treatment of such arrhythmias. The results of this collaborative effort will hopefully yield additional information regarding the question as to whether the self-administration of either of these two agents is desirable when symptoms of acute myocardial infarction are evident but when some time will be required before the patient can be seen by a physician.

b) Chronic Angina Pectoris. We have studied the symptomatic and circulatory effects of a six-week program of intense physical training in patients with angina pectoris due to coronary artery disease. After training the intensity of exercise attained before angina increased 57%. The triple product (TP) of aortic systolic pressure, heart rate, and ejection time was calculated and used as an index of myocardial oxygen consumption ($M\dot{V}O_2$). TP (and presumably $M\dot{V}O_2$) at any level of exercise was less after training, thus accounting for part of the improved exercise capacity. However, after training a higher TP could be achieved before the onset of ischemic pain in over half of the patients. If changes in TP accurately reflect changes in $M\dot{V}O_2$, then the finding that ischemic pain occurred at a higher TP suggests that training, in addition to improving the efficiency of the circulatory response to exercise, might also improve myocardial oxygen delivery.

It has been suggested that a major portion of the beneficial effects of the beta-blockers in treating patients with angina pectoris could be achieved by merely depressing the heart rate response to sympathetic stimulation. Although reports have appeared suggesting that the beta-blocking agent practolol has such effects, we found in dogs that a given reduction in heart rate produced by propranolol and practolol was accompanied by the same negative inotropic response. We also found that while practolol may exert a modest positive inotropic effect, this action is detectable only after full beta-blockade or when beta-receptor stimulation is minimal; such an effect is inapparent if beta-receptor stimulation is increased, which is often the case when beta-blockade is employed clinically. Thus, practolol as used therapeutically does not appear to act on heart rate more selectively than propranolol.

Although many operative attempts have been employed to treat patients with coronary artery disease, no procedure appears to be more promising than the saphenous vein bypass operation. We are currently involved in a collaborative effort with the Surgical Branch to assess the results of bypass as well as to determine the type of anatomic disease that would be most amenable to such a procedure.

Characterization of Fibrinolytic Mechanisms

Fibrinolysis may play a role in retarding the development of coronary artery disease by dissolving fibrinous deposits at the site of endothelial injury. Factors that alter fibrinolysis in man are largely unknown. We have shown previously that the magnitude of the fibrinolytic response to exercise was related to the duration and intensity of exercise, as well as to the time of day it was performed. We also observed that fibrinolytic activity failed to increase normally during intense exercise performed by a group of patients with type IV hyperlipoproteinemia. More recently we have demonstrated that the diurnal augmentation of fibrinolytic activity normally occurring in young subjects is encountered less frequently in older subjects. Patients with type IV hyperlipoproteinemia, a condition associated with the early development of atherosclerosis, show an impairment that is greater than that which can be attributed to age alone. Moreover, patients with coronary artery disease but with normal lipid studies demonstrate smaller diurnal increases than young normal subjects. Although the latter differences can be attributed partly to age-related factors, there is a tendency for further impairment that appears to be independent of age. Whether these defects play a role in the early development of coronary artery disease remains to be determined, as does the cause of the defective response.

Evaluation of Myocardial Function

Several important indices of myocardial performance depend upon accurate and frequent measurement of ventricular volume. To make such measurements feasible, we have developed a semi-automated method for the continuous determination of ventricular volume in man. When volumes determined by the automated method are compared with those obtained by manual planimetry, the correlation coefficient is 0.96; volumes of test objects are accurate to within 6%. This automated technique thus permits rapid and accurate measurement of ventricular volume in all patients having diagnostic left ventriculograms.

CIRCULATORY PHYSIOLOGY AND CLINICAL CARDIOLOGY

We have shown that the heart rate response to baroreceptor mediated enhancement of sympathetic activity is impaired in patients with depressed cardiac function as is the maximum heart rate response to exhausting exercise. Although the etiology of this impairment has not been defined, the defect does not appear to be due either to decreased sensitivity of the adrenergic receptor site or differences in background vagal tone. Its potential for reversibility also is unknown. To more fully characterize this defect, the heart rate responses to 1) baroreceptor enhancement of sympathetic activity and 2) exhausting exercise have been determined in functional class II and III patients about to undergo cardiac surgery. Samples of atrial and left ventricular papillary muscles have been obtained at the time of operation for analysis of norepinephrine content in an attempt to correlate depletion of these stores with the impaired heart rate responses. The preoperative studies will be repeated postoperatively to determine if the anticipated improvement in cardiac function is associated with an improved capacity of the heart to respond to sympathetic stimulation.

Individuals with severe obstruction to outflow of either the right or left ventricles may experience syncope during or immediately after strenuous exercise. In order to clarify the hemodynamic basis for this phenomenon, several circulatory indices were measured while dogs with pulmonary artery constriction exercised to the point of syncope. Pulmonary constriction was achieved by the inflation of a balloon cuff previously implanted around the pulmonary artery. Preliminary results show that syncope is almost always caused by rapidly progressive arterial hypotension rather than by arrhythmia. Decline in cardiac output generally accompanies the fall in blood pressure, suggesting that inadequacy of cardiac output rather than sudden vasodilatation is responsible for the decline in blood pressure which eventuates in circulatory collapse.

The heart rate of anesthetized or alert hyperthyroid animals is increased, as is the intrinsic frequency of contraction of hearts isolated from such animals. However, we have found that the basal heart rates of intact unanesthetized cats are not different from those of normal cats. To explain these differences, we have examined the possibility that the negative chronotropic effect of the vagus is enhanced in hyperthyroid cats. Preliminary results have shown that several cardiac actions of acetylcholine are enhanced in hearts of hyperthyroid cats, findings compatible with the hypothesis that the normal basal heart rate of hyperthyroid animals is due to increased sensitivity of the sinus node to acetylcholine.

The maximal functional capacity of the hearts of patients who have undergone "total correction" of congenital heart defects has been evaluated by catheterization at rest and during intense treadmill exercise. Patients whose atrial septal defects have been completely closed and whose routine catheterization studies are normal may have residual impairment of the cardiac output response to intense upright exercise in the absence of pulmonary arterial hypertension. Patients with corrected tetralogy of Fallot have minimal impairment of their cardiac output response, but may markedly increase their right ventricular outflow gradient and right ventricular pressure during exercise.

Annual Report of the
Section on Clinical Biophysics
Cardiology Branch
National Heart and Lung Institute
July 1, 1970 through June 30, 1971

The activities of this Section have continued along the lines outlined in last year's report. Our principle efforts have centered around two related areas of research: Experimental atherosclerosis and vascular physiology. In experimental atherosclerosis we have completed the first major phase of our program which was designed to examine the detailed topography and histology of spontaneously-occurring and induced atherosclerosis in swine and dogs. We have confirmed the findings of others that atherosclerosis occurs spontaneously in swine, is accelerated by feeding a high-fat, high-cholesterol diet, and is further augmented by inducing the hypothyroid state. We have also confirmed the fact that dogs do not normally develop atherosclerosis unless the hypothyroid state is induced. Moreover, we have found that the porcine atherosclerosis has a histologic picture which closely resembles the disease in human subjects, i.e., it is usually associated with a marked intimal fibromuscular hyperplasia, whereas in dogs the lesions appear more xanthomatous. The cytologic characteristics of the disease process in both species using both a light microscopy and electron microscopy was also found to be consistent with that reported in the literature.

In contrast to the above, certain new observations emerged from our experiments: First it was found that the gross topography of the lesions in early disease was strikingly similar among all groups of animals of both species. The sites of greatest occurrence of lesions were in areas of vessel branch points and their associated entrance regions. The sites of highest lesion incidence were: the lower aortic trifurcations, the sinuses of Valsalva, the aortic arch orifices, the major branch orifices in the upper abdominal aorta, the entrance regions of both the coronary arteries, as well as the main bifurcation and other branch points of the extramural coronary arteries. The presence of grossly apparent fibromuscular intimal thickening in the older animals did not significantly alter the topography of lipid deposition.

Although the over-all topographical distribution of lesions in the different animal preparations and different species were strikingly similar, careful study of the detailed topography at each anatomic site showed interesting, consistent, and different patterns of sudanophilia. A classification scheme was developed so that these patterns could be quantified. Analysis of the topography using this scheme showed that sudanophilia patterns appear to be unique not only with respect to anatomical site but also to the type of animal preparation. Although the underlying causes for these intriguing differences presently remain obscure, they are of obvious etiologic and pathogenic significance, pointing to the presence of strong localizing factors both of a hemodynamic, histologic, and chemical nature. These observations have been incorporated into the experimental design of the second phase of this program and will be pursued as appropriate techniques are developed.

We have also found that lesions which have the histologic appearance of

fatty streaks in many cases go on to develop into classical raised atheromatous lesions indicating that the fatty streak can be an early form of true atherosclerosis. We also observed an inverse relationship between the presence of dense, oriented collagen and lipid deposition. This was seen in two circumstances: at branch points and in pre-existing areas of fibromuscular hyperplasia.

At branch points areas of collagen are laid down in a pattern suggesting that it is in response to chronic stress exposure related to the hydromechanical forces in these regions. For example a dense sheet of collagen is developed over the flow divider of most junctions forming a tough protective sheath buttressing the endothelial cells against the increased forces required to deflect the blood flow at these points. Lipid deposition in these regions of dense collagen is seen only rarely and then only when the serum lipid levels have been elevated to very high values. This may explain the unique detailed topographic distribution of lipid that was described above, i.e., the detailed topographic patterns of sudanophilia may be related to the distribution of pre-existing less permeable fibrous structures.

While fibromuscular proliferation and collagen deposition could also be interpreted as a result of lipid infiltration (i.e., in response to the "toxic" effects of lipid), we have observed a number of instances where lipid deposition and fibromuscular proliferation were observed to occur separately. This suggests, at least under certain circumstances, these processes are independent of one another. For example, lipid infiltration commonly was seen in the absence of fibromuscular changes in dogs with elevated serum lipids, and, conversely, fibromuscular proliferation was frequently observed in the absence of stainable lipid in both euthyroid swine and dogs.

Further evidence for the independence of these processes was found in a study designed to show the potentiating effect on atherogenesis of increased blood flow (shear stress). In these studies arteriovenous shunts were constructed both for the carotid artery and for the iliofemoral artery such that the shunted artery in each case carried about five times as much flow as the contralateral control artery. The shunted iliofemoral artery responded to this increased stress with a marked increase in atherogenesis, whereas the shunted carotid artery responded with marked fibromuscular hyperplasia but no significant increase in lipid deposition. These studies point to the fact the arterial interface does respond to increased hydromechanical forces and depending on local tissue factors can respond with increased fibromuscular hyperplasia, or with increased lipid deposition.

Atheromatous involvement of the media which has been described in man also was observed in both species of animals studied here. Three forms of involvement were observed. The first type of involvement, which is similar to that reported in the literature, occurred in areas of matured atheromatous lesions and appeared to represent a simple spillage of the atheromatous material from the plaque through a broken internal elastic lamella into the medial region. In this situation the lipid had a coarse, granular appearance and appeared to evoke strong tissue reaction in the form of phagocytosis and smooth muscle infiltration. This second type of medial involvement occurred most frequently in elastic arteries and consisted of a homogeneous deposition of oil red O positive material which was diffuse and tended to fill all of

the interlamellar spaces of the wall. This type of involvement appeared to be more physiological in that it did not evoke any apparent tissue response. It was seen most commonly in the ascending aorta and frequently would extend from media to the adventitial capillary bed, suggesting that it might represent an augmented but normal flux of lipoproteins across the arterial wall as a result of hyperlipidemia. The third type of involvement appeared to be similar to the second; however, it usually occurred in areas with the preexisting intimal involvement at the periphery of fibromuscular thickenings and was occasionally associated with some cellular response.

The physiological studies which bear on the foregoing observations have been carried out both in vivo and in vitro. In an effort to demonstrate further that the vascular interface responds to the adjacent hydromechanical forces another study was done in addition to the aforementioned shunt studies. In this study it was observed that the orientation of endothelial nuclear patterns appears to correspond to the adjacent shear stress distribution pointing in the direction of the streamlines of flow. For example, endothelial cells upstream from regions of orifices all tend to point toward the orifice whereas in relatively uniform straight sections of vessel the nuclei tend to have a longitudinally-oriented pattern. One such straight segment is the descending thoracic aorta which invariably has elongated nuclear endothelial nuclei all pointing parallel to the axis of the blood vessel. A segment of the descending thoracic aorta was removed, opened longitudinally, and then the two cut circumferential edges were sewed together to form a tube again, the axis of which now, however, was 90° removed from its original axis, such that the endothelial cells now all point circumferentially. This tubular segment was then resewn into its original bed and the subsequent changes in endothelial cell pattern observed sequentially over the ensuing three weeks. It was found that within three days the endothelial cells begin to turn from their circumferential orientation toward a longitudinal orientation and by ten days all cells are now oriented again in longitudinal direction. This, with the aforementioned A-V shunt data, again shows that the vascular interface is responsive to the adjacent hydrodynamic forces.

A series of in vivo studies have shown that the topography of the flux of Evans blue-tagged albumin appears to be greatest in the same areas that the aforementioned homogeneous oil red-O staining material was greatest. These data taken together with the studies of Duncan who showed the flux of cholesterol also was greatest in the ascending aorta, lend further support to the aforementioned suggestion that this particular type of medial lipid deposition may be related to a normal transmural flux of lipoproteins.

A number of in vitro experiments have been carried out to study the factors influencing protein transport across the vascular interface. A specially designed device, described in detail in previous communications, was used in which the thermal, mechanical, and chemical milieu of the blood vessel could be controlled or measured. In this system it was possible to estimate the mass transport of a given protein species (albumin in this case) as a function of both time and these other variables. It was shown that the excised blood vessel remains "physiologically" normal for periods up to four hours provided it is kept under autologous serum. The salient new findings were: first, a potent barrier to albumin flux esists in a local region associated with the

endothelial cells. Removal or damage of the endothelial cells causes a 100- to 1000-fold increase in the flux of albumin across the vascular interface. Second, transmural pressure differences of up to 280 cm of water pressure do not influence the flux of albumin, either in the presence or absence of endothelial cells. This suggests that protein flux across the arterial wall in the presence of a normal media is not influenced by the pressure drop. Third, the flux of albumin across the interface, however, is greatly increased when the surface is stretched. Finally, a wide variety of solvents, including saline, and particular solutions containing certain polar solvents such as ethyl alcohol and acetone greatly increase the permeability of the interface to protein.

In summary, these studies indicate that the major barrier to albumin (probably also lipoprotein) transport exists locally in the region of the endothelial surface; it depends on the presence of normal endothelial cells; it is weakened either by the elution of some material or by decreasing the oncotic properties of the adjacent fluid; it is decreased by stretching and exposure to increased shear stress; it is not influenced by the transmural pressure itself. These studies are being continued to define the associated transport mechanisms in greater detail.

A number of theories have been proposed to explain the pathogenesis of atheromata. The essence of these can be considered under three major headings; the filtration, the intrinsic and the thrombogenic theories. The filtration theories consider the increased deposition of lipid to be the result of an imbalance between the lipoprotein flux into and out of the intimal tissues. The intrinsic theories consider the increased lipid deposition to be secondary to local alterations in tissue chemistries, such that increased amounts of lipid are being synthesized <u>in situ</u> or that insoluble lipid accumulates from accelerated breakdown of lipoprotein molecules. The thrombogenic theories suggest that the initiating process is the formation of microthrombi on the endothelial surface with secondary changes in local lipid transport and metabolism.

The data presented in this report are not at odds with any of these ideas and in fact tend to reconcile some of the apparent differences in viewpoint. All of these ideas can be related at least in theory to predisposing hydromechanical events. For example, the flux of proteins, including lipoproteins into or out of the animal tissues, can be altered significantly by the state of stress and the associated chemical milieu as was shown in our physiological studies. Moreover, protein synthesis, including the laying down of oriented-structured proteins, such as collagen, has been shown to be related to chronic exposure to stress in studies of bone and tendon by other workers. Furthermore the rate of synthesis of cholesterol by fibroblasts has been shown by Avigan to be increased by prior mechanical agitation. Finally, endothelial injury caused by increased exposure to shearing stress results in increased permeability and influx of serum proteins, as well as an increased affinity for platelet adhesion and fibrin deposition, as shown by previous studies from this laboratory. Thus, when the foregoing theories of atherogenesis are viewed together with the present data they appear entirely compatible. It would be unwise to consider any one of them the sole process leading to the formation of atheroma. The present groups of studies would suggest that each

of these theories probably represents only one component of a complex system of independent and often interacting atherogenic processes, many of which are coupled to associated mechanical events.

The characteristic topographic patterns of lipid deposition and consistent location of fibromuscular intimal thickenings described in these studies point to local factors that act at a level of resolution not previously appreciated. If we are to try to establish correlations between mechanical stresses, local rheologic properties, structural protein patterns, histochemical properties and local tissue biochemistry, we cannot rely upon the relatively gross techniques currently available but instead must evolve a new battery of more sophisticated microtechniques which implies the need for acquiring new talent, special tools, expanded collaborative efforts, contractural help, and an increased budget to accommodate these needs.

Annual Report of the
Section of Pathology
National Heart and Lung Institute
July 1, 1970 to June 30, 1971

This section is concerned with structural changes produced in heart, blood vessels, and lung by cardiovascular and pulmonary diseases. This section works primarily with human "material," much of which is submitted from hospitals and institutions outside the Clinical Center of the National Institutes of Health. The number of hearts submitted to this section have progressively increased: in 1967, the number was 159; in 1970, the number was 298. The submitted specimens are studied routinely by gross and histological means and occasionally by electronmicroscopic means. The number of paraffin blocks prepared in the section's histology unit in 1970 were 3,789. The number of hematoxylin and eosin stains performed was 5,246; the number of special stains, 3,425; and the number of unstained slides, 10,764. Thus, 19,435 slides were cut and 8,671 slides stained during this 12-month period. Approximately 4,000 of the 19,000 slides cut were large (3x1.5 inches). All gross photography performed for this section has been done in the Laboratory of Pathology, National Cancer Institute.

Coronary Heart Disease: A continuing project of this section has been the study of major extra- and intramural coronary arteries of patients dying of acute myocardial infarction. During the past 2 years 92 patients have been studied at necropsy. The 3 major extramural coronary arteries were excised intact, decalcified, sectioned, processed, and 3 histologic sections from each 0.5 cm. segment were examined. Among the 92 patients, 66 had transmural myocardial infarcts, 7 has subendocardial infarcts, and 19 died suddenly (<6 hours) without myocardial necrosis. Thrombi were found in extramural coronary arteries in 33 (50%) of 66 patients with transmural infarcts, in 0 of 7 with endocardial infarcts, in 2 of 19 patients who died suddenly. The number of major extramural coronary arteries (3 per patient) narrowed greater than 75% by old atherosclerotic plaques was 2.4 in the transmural cases, 2.0 in the subendocardial cases, and 2.3 in the sudden death patients. Of the many thousands of slides of coronary arteries examined in the 92 patients, only 3 sections showed normal coronary arteries. Although the degree of luminal narrowing varies considerably, coronary arterial atherosclerosis is a diffuse disease. The lumens of the coronary arteries distal to sites of thrombosis were >75% narrowed by old plaques in 33 of the 35 patients with thrombi. The coronary artery distal to a thrombus has never been studied previously. Since thrombi were rare in sudden death cases, and since they occurred only in the coronary arteries of patients who already had severely narrowed vessels, it is believed that thrombi are consequences rather than causes of myocardial infarcts. Thrombi were observed almost exclusively in patients dying with features of the pump failure syndrome (shock or severe congestive heart failure).

Prosthetic Cardiac Valves: A continuing effort of this section has been the morphologic evaluation of hearts and other organs of patients dying after replacement of one or more cardiac valves with prosthesis. From 1963 through 1970, 181 patients died after valve replacement: 107 within 2 months of

operation, and 74 at later periods up to 80 months. Among the causes of death early, prosthetic dysfunction was responsible in 30%, no anatomic cause was found in 28%, bleeding and technical mishaps 19%, infection 4%, and miscellaneous causes 5%. Among the 74 patients who died late, prosthetic dysfunction accounted for death in 37% and factors secondarily related to prostheses in 24%. All patients who died late following aortic valve replacement had intimal fibrous proliferation in the aortic roots. Whether or not the coronary arterial ostia will become narrowed with time following aortic valve replacement remains to be seen. The degree of intimal proliferation in the ascending aorta in patients who died late was proportional to the degree of renal hemosiderosis, indicating that the aortic lesion is due to turbulence of blood traversing the aortic valve and that this turbulence causes intravascular hemolysis which is apparent in the kidney by deposition of iron in renal tubules. Whether or not the metallic ball now used in Starr-Edwards prostheses will hold up over many years remains to be seen. There is suggested evidence now that the cloth-covered struts will show evidence of wear after about 3 years. Although additional years of life have been provided to many critically ill patients from severe valvular heart disease by cardiac prostheses, it is apparent 10 years after valve replacement began that the ideal cardiac valve is not presently available. It would appear from this study that the caged-ball rigid frame prostheses are simply not capable of functioning properly in a few selected hearts with either small left ventricular cavities or small aortas.

Aortic Valve Disease: Among the congenital malformations of the aortic valve, the least understood and the least well recognized is the unicuspid unicommissural valve. This valve is the most common cause of aortic stenosis during the first year of life, but its recognition in adults has been infrequent. Twenty-one adult patients found at necropsy or surgery to have unicommissural valves were studied. Although this basic valve structure may render it inherently stenotic, the long duration of the murmur and the late age of onset of symptoms of left ventricular outflow obstruction in these patients strongly suggest that stenosis, at least in part, is acquired. Since these valves are characterized by only one commissure, valvotomy is hazardous and valve replacement appears indicated if operation is indicated.

Endocarditis: A major project of this section during the past year has been the study of necropsy patients with infective (I) and non-infective endocarditis (E). Of 55 necropsy patients with active valvular IE, 58% had vegetations on previously anatomically normal valves. Predisposing factors allowing entrance of virulent or unusual organisms or alteration of host defense mechanisms occurred in 70% of patients with IE on previously normal valves. Valvular dysfunction occurred in 70% of patients with IE on previously normal valves. Valvular dysfunction occurred in 70% of the 55 patients, causing congestive heart failure in all. Myocardial lesions were present in 92% of the 39 patients in whom multiple histologic sections of myocardium were examined. A new observation was the finding of papillary muscle necrosis in 75% of patients; mitral regurgitation occurred as a result of this necrosis in only one. Since myocardial inflammation was focal in all but 2 patients myocardial lesions are not believed to be a primary cause of congestive heart failure in patients with IE.

Twenty-nine necropsy patients with healed valvular IE were reviewed. Active IE had occurred on anatomically abnormal valves in 24 patients, 9 of whom had congenitally bicuspid aortic valves. All 29 patients had evidence of valvular dysfunction during and after the active IE and 27 had overt congestive heart failure. Active IE was the sole or contributory cause of valvular dysfunction in 23 of the 29 patients. The presence of fibrosis of the papillary muscles in 83% of the 29 patients supports the view that this lesion is the result of healed papillary muscle necrosis, observed frequently in the patients with active IE.

Five patients with active IE confined to mural endocardium were studied. This type of endocarditis is unique. Each patient had an underlying malignant disease and the causative organism in each was a fungus. The vegetations were located on right ventricular endocardium in 3 patients and on left atrial endocardium in 2. In each, the mural IE was not primary but rather the result of extension of an infective process originating at another site. No evidence of cardiac dysfunction occurred in any of the 5 patients.

The clinical and necropsy features of 45 patients with active non-infective ("marantic") endocarditis also were studied. This type of endocarditis is characterized by the lack of organisms demonstrable on histologic section of the vegetation. Vegetations occurred on anatomically normal valves in 40 patients and in functionally normal valves in 44 patients. Malignancy was present in 39 patients. A precordial murmur occurred in 55% of the patients. Systemic emboli to the brain caused death in 6 patients.

Congenital Aneurysm of the Ductus Arteriosus: Although patent ductus arteriosus is a well-recognized congenital malformation, aneurysm of this structure rarely has been appreciated. An aneurysm at this site, however, was observed in a 2-month-old child. The literature in 60 previously described patients with ductal aneurysm was reviewed. It is apparent that the aortic end of the ductal aneurysm is always patent and that the pulmonary arterial end may or may not be patent. Nearly half of the ductal aneurysms develop complications (rupture, embolism or infection) and therefore operative resection appears warranted when this diagnosis is established.

Papillary Muscle Dysfunction: A unique opportunity presented itself when a 71-year-old man was admitted to a local hospital with what turned out to be a silent acute myocardial infarction. This patient presented evidence of severe congestive heart failure, but since the cause of the heart failure was unknown cardiac catheterization was undertaken. At the first study, when no precordial murmur was apparent, hemodynamics indicated that the mitral valve functioned abnormally. Two weeks later, when a loud murmur of mitral regurgitation was present, hemodynamics reconfirmed severe mitral regurgitation. The uniqueness of this case lies in the fact that, for the first time, serial cardiac catheterization was performed in a patient during the period when papillary muscle necrosis was taking place. Also, hemodynamic mitral regurgitation was demonstrated before auscultatory mitral regurgitation was apparent.

Electron Microscopy

The electron microscopic unit of this section, which became fully

operational in April 1970, yielded several studies. The spleen in type I hyperlipoproteinemia was studied ultrastructurally. Foam cells were observed that contained a material identified as ceroid. The ceroid was organized in the form of granules and the process of formation of these granules was described. The ceroid was considered to represent non-digestible end products of the metabolism of chylomicrons taken up by macrophages in splenic sinusoids. Several cardiac myxomas were studied ultrastructurally. The electron microscopic observations support the concept that the tumor cells are derived from endocardial endothelial cells. Ultrastructural observations were made in 12 patients who had left ventricular outflow myocardium excised at time of operation. Each of these patients had idiopathic hypertrophic subaortic stenosis and electron microscopy disclosed that the ultrastructural features of this entity are distinctive and are characterized by severe disorientation of myocardial fibers. The patterns observed in the muscle excised from the left ventricular outflow tract were compared to muscle observed at left ventricular apex and the severe disorientation pattern was not observed in the apical biopsies. The features of the muscle in the left ventricular outflow tract are similar to muscle observed in newborns and suggest that this disorientation, and thus the basic disease, is present from the time of birth.

Ultrastructural studies were made on the acute and chronic effects of normothermic anoxia on canine hearts. All 16 dogs subjected to 45 minutes of cardiac anoxia showed extensive myocardial damage ultrastructurally whereas all 7 dogs subjected to 3 minutes of cardiac anoxia showed only minimal myocardial damage. These observations would not have been apparent on light microscopy whereas they were clearly apparent by electron microscopy. The effects of hyperosmotic perfusate on ultrastructure and function of the isolated canine heart were studied. Eleven dogs were studied: 5 were perfused with filtered plasma, and 6 with filtered plasma-dextran. Interstitial edema, swelling of sarcoplasmic reticulum, and mitochondrial damage were observed in each of the 5 hearts perfused by filtered plasma. In contrast, interstitial edema was absent in each of the 6 hearts perfused by filtered plasma-dextran and swelling of sarcoplasmic reticulum and mitochondrial damage occurred in only 2. Thus, the osmolarity of the perfusate is important in preventing edema in perfused hearts.

A continuing study of this section has been the ultrastructure of myocardium removed at biopsy in patients with primary myocardial disease. The cardiac biopsies are being done at either D.C. General Hospital or at the Veterans Administration Hospital in Washington, D.C. Observations at this point indicate that mitochondrial alterations, swelling of sarcoplasmic reticulum and dilatation of the transverse tubular system are the main alterations present. These alterations, however, are not specific for primary myocardial disease. None of the observations has proved useful from a diagnostic standpoint.

Serial No. NHLI-1
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Indivudual Project Report
July 1, 1970 through June 30, 1971

Project Title: Potentiation of the Inotropic Effects of Norepinephrine, Glucagon, and Dibutyryl Cyclic AMP by Theophylline

Previous Serial Number: None

Principal Investigator: C. Lynn Skelton, M. D.

Other Investigators: Melvin L. Marcus, M. D.
Fred E. Karch
Thomas J. Hougen
Stephen E. Epstein, M. D.

Cooperating Units: None

Project Description: The positive inotropic response to catecholamines and glucagon has been postulated to result from an increase in the intracellular level of cyclic AMP, produced by activation of adenyl cyclase. If this hypothesis is valid, inhibition of phosphodiesterase, the enzyme responsible for inactivating cyclic AMP, would be expected to enhance the cardiac effects of these agents. We therefore examined the effects of theophylline, an effective inhibitor of phosphodiesterase, on the isometric response of cat papillary muscles to varying concentrations of norepinephrine (10^{-11} to 10^{-6} M), glucagon (10^{-8} to 10^{-5}), and of dibutyryl cyclic AMP (DBC) (10^{-8} to 10^{-3} M), a more lipid soluble derivative of cyclic AMP which is thought to mimic the intracellular effects of cyclic AMP. At a theophylline concentration (2.5×10^{-4} M) which caused little increase in baseline contractile function (avg. less than 10%), a marked potentiation of the inotropic effects of norepinephrine, glucagon and DBC was observed. Thus, the concentration of norepinephrine producing half maximal activity decreased from 1×10^{-7} M to 1×10^{-8}M: that of DBC decreased from 8×10^{-4} to 4×10^{-4} M. In addition, the peak tension development produced by glucagon increased from $0.7 \pm .25$ g/mm^2 to $3.0 \pm .72$ g/mm^2 ($p < .01$). In contrast, theophylline did not alter the inotropic effects of calcium (2.5×10^{-3} - 1.0×10^{-2}), an agent not believed to exert its inotropic effects through the cyclic AMP system. These findings provide further evidence that the inotropic effects of norepinephrine, glucagon, and DBC are mediated via increases in the intracellular level of cyclic AMP.

Proposed Course of Project: Completed

Honors and Awards: None

Publication: Submitted for publication

Serial No. NHLI-2(c)
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Comparison of Left Ventricular and Pulmonary Arterial Injection Sites in the Determination of Cardiac Output by the Indicator Dilution Technique

Previous Serial No.: None

Principal Investigator: Richard L. Shepherd, M.D.

Other Investigators: D. Luke Glancy, M.D.
Lawrence M. Higgs, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: The validity of the indicator dilution technique for measuring cardiac output has been established by comparing the values obtained with those determined simultaneously by the Fick method. In such studies indicator was injected in the lesser circulation and sampled in a systemic artery. Because the adequacy of the left ventricle as a mixing chamber has been questioned, left ventricular injections seldom have been used. This study was undertaken to assess the accuracy and reproducibility of cardiac output measurements made from left ventricular indicator dilution curves.

In 58 patients with a variety of hemodynamic abnormalities, four indicator dilution curves were inscribed in rapid succession alternating the site of injection between pulmonary artery and left ventricle and using a single systemic arterial sampling site. Indocyanine green was the indicator.

The average cardiac output calculated using the left ventricular curves exceeded that using the pulmonary arterial curves by 0.037 L/min (standard error $\pm$ 0.052 L/min). The estimated standard error of a left ventricular measurement of cardiac output was 0.41 L/min and that of a pulmonary arterial measurement was 0.35 L/min. Thus, cardiac output determined from left ventricular curves did not differ significantly from determinations made from pulmonary arterial curves, and the reproducibility of the two methods was essentially the same.

Data from individual patients with the largest differences between cardiac output determinations by the two methods suggest that in the presence of severe mitral regurgitation left ventricular curves tend to overestimate output while pulmonary arterial curves tend to underestimate output. We

conclude that left ventricular indicator dilution curves give accurate and reproducible measurements of cardiac output except in occasional patients with severe mitral regurgitation.

Proposed Course: Completed

Honors and Awards: None

Publications: In preparation.

Serial No. NHLI-3(c)
1. Cardiology
2. Cardiovascular Diagnosis
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Acquired Right Ventricular Infundibular Stenosis in Patients with Ventricular Septal Defect

Previous Serial Number: None

Principal Investigator: Richard L. Shepherd, M.D.

Other Investigators: D. Luke Glancy, M.D.
Richard B. Jaffe, M.D.
Joseph Perloff, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Radiology Dept., Clinical Center; Division of Cardiology, Dept. of Medicine, Georgetown Univ. School of Medicine

Project Description: With the possible exception of bicuspid aortic valve, ventricular septal defect (VSD) is the commonest congenital cardiovascular malformation seen in children. Although many patients with this defect die in infancy of left ventricular failure or develop severe pulmonary vascular disease and die as young adults, the drop-out rate due to these complications is not sufficient to explain the observation that it is relatively uncommon to see an adult with uncomplicated VSD. The observation that many patients with a VSD experience spontaneous closure of the defect explained the discrepancy in prevalence of this entity in children and adults. However, clinical studies have suggested that development of right ventricular obstruction is another possible cause for the infrequency with which isolated VSD is seen in adults. By serial hemodynamic studies we have recently documented the development of acquired right ventricular infundibular stenosis in five children with VSD.

When first studied at ages six months to four years, each child had pulmonary arterial hypertension, a trivial or absent pressure gradient across the right ventricular outflow tract, and a large left-to-right shunt at ventricular level. When restudied 1 1/2 to five years later, each was found to have a normal pulmonary arterial pressure, a large pressure gradient across the right ventricular infundibulum, and a reduced left-to-right shunt. Two of the patients had large right-to-left shunts at ventricular level, and like patients with tetralogy of Fallot presented on their second admission with cyanosis and squatting. Both have undergone corrective operations.

This study thus demonstrates that severe right ventricular infundibular stenosis can develop in children with uncomplicated VSD. It also indicates that while the acquisition of right ventricular infundibular stenosis is beneficial in some patients with VSD (since pulmonary arterial pressure and

flow are thereby reduced), the infundibular narrowing can become excessive and result in a physiologic situation identical to that seen in Fallot's tetralogy.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: In preparation

Serial No. NHLI-4(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hemodynamic Assessment of Kay Shiley Prosthetic Valves

Previous Serial Number: None

Principal Investigator: Richard L. Shepherd, M. D.

Other Investigators: D. Luke Glancy, M. D.
R. Reis, M. D.
Stephen E. Epstein, M. D.
Andrew G. Morrow, M. D.

Cooperating Units: Clinic of Surgery

Project Description: Previous studies have assessed the in vivo hemodynamic function of Starr Edwards valve prostheses but there is no information available relating to the performance of the low profile disc type (Kay Shiley) valve prosthesis.

Since January 1966, fifty-one Kay Shiley valves have been used to replace diseased mitral and/or tricuspid valves in 48 patients. Twenty-three of the 48 patients had mitral valve replacement; three of the 23 patients also had tricuspid annuloplasties and two others had myotomy for IHSS. In 18 of the 48 patients both mitral and tricuspid valves were replaced; 4 of the 18 patients had Kay Shiley protheses in both mitral and tricuspid areas and the others had a Starr Edwards mitral prothesis and a Kay Shiley tricuspid prosthesis. Three patients had Starr Edwards aortic protheses and Kay Shiley mitral prosthesis and 4 patients had Starr Edwards aortic and mitral prostheses and Kay Shiley triscupid prostheses.

There have been 22 postoperative deaths and 8 patients who have been lost to follow up. There are 18 survivors who have had postoperative catheterizations. In patients with tricuspid valve replacement, the right atrial mean pressure was 8 mm Hg or more in every case. In patients with mitral valve replacement, the left atrial mean pressure was 18 mm Hg (average). Calculated valve areas in vivo were 66 - 75% of the in vitro valve areas provided by the manufacturer.

The operative mortality in this group of patients is considerably higher than the mortality in this institution using the caged ball prosthesis. The reasons for the higher mortality are not clear. Many of these patients had more than one valve replaced and the low profile valve was usually selected when the ventricular cavity would not accept a caged ball prosthesis. As has been found in the caged ball prosthesis, the Kay-Shiley prosthesis causes mild to moderate obstruction to atrial emptying.

Serial No. NHLI-4(c)

PHS-NIH
Indivudual Project Report
July 1, 1970 through June 30, 1971

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHLI-5(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Reversibility of Impaired Heart Rate Response to Sympathetic Stimulation in Patients with Cardiac Decompensation

Previous Serial Number: None

Principal Investigator: Douglas R. Rosing, M. D.

Other Investigators: G. David Beiser, M. D.
David R. Redwood, M. D.
Eldon R. Smith, M. D.
Stephen E. Epstein, M. D.

Cooperating Unit: None

Project Description: We have shown that the heart rate response to baroreceptor mediated enhancement of sympathetic activity is impaired in patients with depressed cardiac function, as is the maximal heart rate response to exhausting exercise. Although the etiology of this impairment has not been defined, the defect does not appear to be due to either decreased sensitivity of the adrenergic receptor site or differences in background vagal tone. Its potential for reversibility also is unknown. To more fully characterize this defect, the heart rate responses to 1) baroreceptor enhancement of sympathetic activity produced by lowering mean arterial pressure with nitroglycerin and 2) exhausting exercise have been determined in functional class II and III patients about to undergo cardiac surgery. Samples of atrial and left ventricular papillary muscles have been obtained at the time of operation. These samples will be analyzed for norepinephrine content in an attempt to correlate depletion of these stores with the impaired heart rate response. In addition, the preoperative studies will be repeated after operation to determine if the anticipated improvement in cardiac function results in an improved heart rate response to sympathetic stimulation.

Proposed Course of Project: As outlined above

Honors and Awards: None

Publications: None

Serial No. NHLI-6(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Aging, Type IV Hyperlipoproteinemia, and Coronary Artery Disease on the Diurnal Pattern of Fibrinolytic Activity

Previous Serial Number: NHLI - 29(c)

Principal Investigator: Douglas R. Rosing, M.D.

Other Investigators: Pieter Brakman, M.D.
David R. Redwood, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: The James F. Mitchell Foundation, Washington, D.C.

Project Description: The fibrinolytic system is important in maintaining an equilibrium between fibrin deposition and dissolution, and it has been postulated that a defect in this system may play a role in the pathogenesis of arteriosclerosis. Therefore diurnal patterns of plasma euglobulin fibrinolytic activity, estimated by the Astrup fibrin plate method and expressed as mm^2 lysis, were determined in 1) young normal subjects - mean age 20; 2) older normals - mean age 44; 3) patients with type IV hyperlipoproteinemia - mean age 44; and 4) patients with coronary artery disease and normal lipid studies - mean age 52. Because fibrinolytic activity gradually increases during the day in young normal subjects at bed rest and peaks between 5 and 8 p.m., samples collected at 8 a.m., 5 p.m., and 8 p.m. were selected to characterize and compare the four groups. Mean peak fibrinolytic activity was also compared among the groups. At 5 p.m. fibrinolytic activity in 18/19 young normals increased at least 75 mm^2; however, only 13/24 older normals ($P < .05$), 5/20 patients with endogenous hypertriglyceridemia, and 3/16 patients with coronary artery disease demonstrated similar increase. At 8 p.m. fibrinolytic activity in 19/19 young normals increased at least 100 mm^2; only 14/24 older normals ($P < .005$), 3/20 patients with triglyceride abnormalities, and 6/16 patients with coronary artery disease exhibited this increase. The difference between the <u>increases</u> in fibrinolytic activity in the age-matched older normals and type IV patients were statistically significant at 8 p.m. but not at 5 p.m. The differences between the older normals and coronary artery disease patients were not statistically significant. <u>Absolute values</u> of mean peak fibrinolytic activity were: young normals = 254 ± 14 mm^2, older normals = 202 ± 22 mm^2, type IV patients 137 ± 14 mm^2, and patients with coronary artery disease 164 ± 17 mm^2. The differences in absolute values between the young and older normals ($P < .05$), and the older normals and type IV patients ($P < .01$) were statistically significant, but the differences between the older normals and patients with

coronary artery disease did not achieve statistical significance. Thus, the data demonstrate that the diurnal augmentation of fibrinolytic activity normally occurring in young normal subjects is encountered less frequently in older subjects. Patients with type IV hyperlipoproteinemia, a condition which has been associated with the early development of atherosclerosis, show an impairment that is greater than that which can be attributed to age alone. Patients with coronary artery disease but with normal lipid studies demonstrate smaller diurnal increases than older normal subjects, although the differences are not statistically significant.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHLI-7
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Indivudual Project Report
July 1, 1970 through June 30, 1971

Project Title: Propranolol in the Treatment of Arrhythmias Occurring During Acute Myocardial Ischemia in Conscious Dog

Previous Serial Number: None

Principal Investigator: Douglas R. Rosing, M. D.

Other Investigators: James Talano, M. D.
Richard Karsh, M. D.
G. David Beiser, M. D.
Stephen E. Epstein, M. D.

Cooperating Units: None

Project Description: Approximately 40% of patients who suffer an acute myocardial infarction die, and 2/3 of these deaths occur within the first few hours, before the patient is admitted to a hospital. Since the mechanism of death under such circumstances is probably due to cardiac arrhythmias, survival following acute myocardial infraction might be increased by the initiation of a program in which high risk individuals receive an effective anti-arrhythmic agent on a chronic, prophylactic basis or in certain acute situations where there is a strong suspicion that an acute myocardial infarction has occurred. Propranolol, administered chronically to patients with angina pectoris, has antiarrhythmic effects and has been shown to decrease the size of ischemic areas after acute coronary occlusion in anesthetized dogs. This drug might therefore prove of benefit to the patient suffering an acute myocardial infarction. On the other hand, since bradycardia decreases the fibrillation threshold of the myocardium and favors the development of re-entry type arrhythmias, the decrease in heart rate that occurs with propranolol administration may predispose to the development of malignant arrhythmias.

To determine whether propranolol has a beneficial or deleterious effect during acute myocardial infarction on arrhythmias, we are studying its effects during acute ischemia produced in closed chest conscious dogs. This is accomplished by emplacing inflatable balloons around the left anterior descending coronary arteries in dogs as a first stage procedure. At least one week after recovery from operation, acute myocardial ischemia is produced in the conscious dog by inflating the balloon and thereby occluding the coronary artery. Stable arrhythmias that develop are treated with .05 mg/kg doses of intravenous propranolol administered every five to ten minutes.

In six dogs that developed ventricular arrhythmias after coronary occlusion, propranolol had no consistent beneficial or detrimental effects.

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In three dogs that had marked ST segment changes but no consistent arrhythmia after occlusion, administration of propranolol did not produce arrhythmias. These preliminary results suggest that while propranolol administered chronically to patients with angina pectoris may not have any detrimental effects if acute ischemia occurs, there is also little likelihood that the drug will prevent arrhythmias occurring during such episodes.

Proposed Course of Project: 1) The number of dogs studied during occlusion of the coronary artery will be increased; 2) the effect of propranolol on arrhythmias which develop after release of coronary artery occlusion will be studied (these arrhythmias are often more malignant than occlusion arrhythmias); 3) the effect of practolol, a "cardio-specific" beta receptor blocking agent with little tendency to lower resting heart rate, will be evaluated.

Honors and Awards: None

Publications: None

Serial No. NHLI-8(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effects of Training in Patients with Coronary Artery Disease and Angina Pectoris

Previous Serial Number: NHLI-25(c)

Principal Investigator: David R. Redwood, M.D.

Other Investigators: Douglas R. Rosing, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: In the last few years there has been increasing awareness of the beneficial effects of exercise in patients with coronary artery disease. Thus, several recent studies have shown that physical training increases exercise performance in patients with angina and that at least part of this improvement is due to a more efficient circulatory response to exercise such that myocardial oxygen demands ($M\dot{V}O_2$) for any given intensity of exercise presumably are reduced. Nevertheless, it is not known whether training also leads to an enhanced capacity of the coronary vessels to deliver oxygen.

We therefore studied the effects of a six-week program of intense training in seven patients with angina pectoris due to coronary artery disease. Aortic systolic pressure, heart rate, and ejection time were recorded continuously during upright bicycle exercise before, at 3 weeks, and at the conclusion of training. After six weeks exercise capacity markedly increased: time to onset of angina rose an average of 6.8 ± 1.5 minutes ($P < .01$) and the intensity of exercise (measured by total body O_2 consumption) attained before angina increased $57 \pm 19\%$ ($P < .005$). The greatest change in exercise capacity occurred during the first 3 weeks of training. The triple product (TP) of aortic systolic pressure, heart rate, and ejection time was calculated and used as an index of $M\dot{V}O_2$. TP (and presumably $M\dot{V}O_2$) at any level of exercise was less after training, thus accounting for part of the improved exercise capacity. However, after training a higher TP could be achieved before the onset of ischemic pain (4885 vs. 4290, $P < .05$). If changes in TP accurately reflect changes in $M\dot{V}O_2$, then the finding that ischemic pain occurred at a higher TP suggests that training, in addition to improving the efficiency of the circulatory response to exercise, might also improve myocardial oxygen delivery.

Proposed Course of Project: Completed.

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHLI-9
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effects of Atropine on the Degree of Myocardial Ischemia During Coronary Occlusion in the Conscious Dog

Previous Serial Number: None

Principal Investigator: David R. Redwood, M.D.

Other Investigators: Eldon R. Smith, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Atropine is frequently employed in patients with acute myocardial ischemia to treat bradycardia-induced arrhythmias, and we have shown experimentally that atropine is effective in treating arrhythmias occurring during the onset of myocardial infarction. It is not known, however, whether the atropine-induced increase in heart rate has any deleterious effect on the degree of myocardial ischemia. To avoid the tachycardia present in open-chest anesthetized dogs, we studied the S-T segment response (obtained from 10 - 12 implanted intramyocardial electrodes) to repeated five-minute occlusions of the left anterior descending coronary artery at hourly intervals in closed-chest conscious dogs in which we previously had implanted an inflatable balloon around the left anterior descending artery. Occlusions were conducted in random order 1) at control heart rate (avg. 95), 2) with atropine pretreatment (.005 - .05 mg/kg/iv), and 3) during atrial pacing. Repeated control occlusions caused no change in degree of S-T response. When compared to control occlusion there was a significant correlation between the per cent increase in heart rate produced by atropine and per cent increase in total S-T elevation ($y = 0.89 x + 5.00$, $r = .95$, $P < .001$). There was no significant difference between the increase in S-T elevation with occlusion following atropine and that following occlusion during rate-matched atrial pacing.

To determine if an increase in heart rate from even slower control rates (as might occur in some patients with acute myocardial infarction) also was deleterious, various degrees of bradycardia were induced by vagal stimulation during episodes of myocardial ischemia. Similar results were obtained: i.e., there was a direct relationship between increases in heart rate from 40 to 95 beats/min and increases in S-T segment elevation.

We conclude that if the degree of S-T elevation reflects severity or extent of ischemia, increases in rate in experimentally produced acute myocardial ischemia are associated with proportional increases in the degree of myocardial ischemia. Thus, when atropine is administered to control bradycardiac-induced arrhythmias during acute myocardial ischemia, the lowest effective dose should be used and excessive increases in rate avoided.

Serial No. NHLI-9

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Serial No. NHLI-10
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Role of Cyclic AMP in Myocardial Contractility

Previous Serial Number: None

Principal Investigators: Melvin L. Marcus, M. D.
Leonard E. Grauer, M. D.

Other Investigators: Gopal Krishna, Ph. D.
Stephen E. Epstein, M. D.

Cooperating Units: Laboratory of Clinical Pharmacology

Project Description: The effects of catecholamines on myocardial contractility are thought to be mediated via increases in the intracellular level of cyclic AMP which result from stimulation of adenyl cyclase. In an attempt to examine this hypothesis more thoroughly, the effects of norepinephrine on myocardial contractility and on tissue levels of cyclic AMP were studied employing the isometrically contracting cat papillary muscle. All experiments were performed at 30°C with the muscles at the peak of their length-tension curve (L_{max}). The effects of two concentrations of norepinephrine (1×10^{-4} and 1×10^{-6}) were studied. At each concentration, the effects of norepinephrine. The papillary muscles were then quickly frozen with cold isopentane, measured, weighed, and assayed for cyclic AMP by the Gilmore method.

Control levels of cyclic AMP in the cat papillary muscle were found to be 1.7 ± 0.1 pcM/mg tissue. A concentration of 1×10^{-4} norepinephrine produced an increase in both myocardial contractility and cyclic AMP within 5 seconds. A concentration of 1×10^{-6} norepinephrine had a positive inotropic effect within 10 seconds, but a significant increase in cyclic AMP concentration could not be demonstrated until 20 seconds had elapsed.

There are several explanations for the dissociation of the inotropic and enzymatic effects of norepinephrine found in these preliminary studies. It is possible that activation of the adenyl cyclase-cyclic AMP system and enhancement of contractility are coincidental and causally unrelated events. On the other hand, it is possible that our technique is not sensitive enough to detect small changes in tissue levels of cyclic AMP which nevertheless may be physiologically significant. It is also possible that the adenyl cyclase-cyclic AMP system is responsible for mediating several intracellular functions, each function being compartmentalized into a separate pool. Thus, each pool may be sufficiently small so that assaying tissues for total cyclic levels becomes too insensitive a technique to discern, for example, changes in the pool specifically responsible for altering myocardial contractility.

Serial No. NHLI-10

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Serial No. NHLI-11(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Completely Automated Video-Tracking Technique for the Determination of Dynamic Changes in Ventricular Volume

Previous Serial Number: NHLI-22(c)

Principal Investigator: Melvin L. Marcus, M.D.

Other Investigators: William H. Schuette
Willard Whitehouse
James Bailey, M.D.
D. Luke Glancy, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Biomedical Engineering and Instrumentation Branch, Division of Research Services;
Laboratory of Applied Studies, Division of Computer Research and Technology;
Administrative Branch, Clinical Center

Project Description: Several important indices of myocardial performance depend upon accurate and frequent measurement of ventricular volume. Studies employing such measurements have been limited because of the difficulty of manually measuring and calculating volumes frequently enough to obtain meaningful data. We therefore have developed a completely automated method for determination of ventricular volume in man. Left ventricular cineangiograms (16 mm) taken in the RAO position at 60 frames/sec are projected with a flickerless projector onto a Plumbicon television camera. A second television camera is used for masking out noncontributory portions of the film and for shading selected areas to facilitate accurate recognition of the opacified chamber. An electronic video-tracking device then simultaneously determines the area and the maximum length of the opacified chamber in each cine frame; these data are recorded as analog signals on magnetic tape. Volumes are calculated by computer and plotted against time. When volumes determined by this automated method are compared with those obtained by manual planimetry the correlation coefficient is $r = .96$; volumes of test objects (20-360 cc) are accurate to within $6.0 \pm 3.6\%$. This automated technique thus permits rapid and accurate measurement of ventricular volume in all patients having diagnostic left ventriculograms.

Proposed Course of Project: Completed
Honors and Awards: None
Publications: Manuscript submitted for publication.

Serial No. NHLI-12
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Action of the Inotropic Effects of Theophylline

Previous Serial Number: None

Principal Investigator: Melvin L. Marcus, M. D.

Other Investigators: C. Lynn Skelton, M. D.
Leonard E. Grauer, M. D.
Gopal Krishna, Ph. D.
Stephen E. Epstein, M. D.

Cooperating Units: Laboratory of Chemical Pharmacology

Project Description: Theophylline is thought to exert its positive inotropic effect on the heart by increasing the intracellular levels of cyclic 3',5'-AMP through inhibition of phosphodiesterase. To investigate this hypothesis, the effects of theophylline on the contractile function of isolated cat papillary muscles were studied. In isotonic experiments, theophylline (2.5×10^{-3}M) caused a marked shift in the force-velocity curve upward and to the right from control levels. In quick release experiments, the modulus of series elasticity was unchanged by 2.4×10^{-3}M theophylline. In isometric experiments, theophylline (2.5×10^{-6} - 2.5×10^{-3}M) caused concentration related increases in tension and rate of tension development that averaged 2.8 ± 0.3 gm/mm^2 ($P < 0.001$) and 9.7 ± 1.4 gm/mm^2/sec ($P < 0.001$) at the peak concentration. Unlike norepinephrine and dibutyryl cyclic AMP (agents believed to work through the cyclic AMP system), time to peak tension development (TTP) was increased. Propranolol (1×10^{-6}M) caused a shift in the concentration-response curve to the right, and prior reserpinization decreased the peak inotropic effect of theophylline by more than 40%. Therefore, the inotropic effects of theophylline are partly due to a direct effect and partly to norepinephrine release. Since theophylline lengthens TTP, its direct inotropic effects either must not be mediated by cyclic AMP, or if they are, then the drug must have additional effects on the contractile mechanisms.

Further investigations have involved the effects of theophylline on the intracellular levels of cyclic AMP in the papillary muscle. The basal level of cyclic AMP in the papillary muscle is 1.7 ± 0.1 pcM/mgm. Theophylline 1×10^{-3}M increased tension in the papillary muscle by approximately 50% but had no significant effect on the intracellular level of cyclic AMP. These data suggest that the inotropic effects of theophylline are probably not mediated by changes in the intracellular level of cyclic AMP.

Proposed Course of Project: Continuing investigation of the effects of theophylline on cyclic AMP levels.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Honors and Awards: None

Publications: None

Serial No. NHLI-13
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzyme and Receptor Activities of Subcellular Systems Isolated from Ischemic Rabbit Hearts

Previous Serial Number: None

Principal Investigator: George E. Lindenmayer, M. D., Ph. D.

Other Investigator: Stephen E. Epstein, M. D.

Cooperating Units: None

Project Description: The present investigation was designed to determine the time-dependent sequence of irreversible subcellular changes in the ischemic myocardium. Male rabbits were sacrificed by cervical dislocation (CD) and the hearts were left in situ for varying times. After CD, no respirations were observed, cardiac contractions ceased within 5 minutes, and body temperature dropped about 5°C in the 150 minutes after CD. Although this model does not represent pure ischemia (since hypoxia probably predominates until cardiac arrest), it is a simple, reproducible one which will permit us to select subcellular systems for study in more physiologic models of pure ischemia. Preliminary results already have demonstrated that subcellular systems differ in their sensitivity to ischemia. For example, Na^+, K^+-ATPase is remarkably stable. Activities 150 minutes after CD were 90% of values obtained 1 minute after CD. Conversely, mitochondrial preparations (isolated in KCl-EDTA medium) showed rapid loss of respiratory control (i.e. 50% decrease within 15 minutes after CD) and rates of oxygen consumption (State 3). The sensitivities of these systems to appropriate ligands will be determined (e.g. Km's for Mg·ATP, sodium and potassium, and Ki for ouabain of Na^+, K^+-ATPase preparations). Other subcellular systems that will be examined are adenyl cyclase, myofibrils, calcium binding by membranes and lysosomes. Eventually, components of ischemia (e.g. hypoxia, substrate lack, etc.) will be analyzed with respect to subcellular changes. An attempt also will be made to correlate ischemic-induced reversible and irreversible changes in myocardial contractility with subcellular functional aberrations.

The eventual goals we hope to achieve with this approach to the study of myocardial ischemia are to determine the subcellular mechanisms responsible for impaired myocardial function during ischemia, and to develop techniques whereby ischemia-induced subcellular injury, and therefore physiologic function, can be minized.

Proposed Course of Project: Outlined in abstract; now in progress.

Honors and Awards: None

Publications: None

Serial No. NHLI-14
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Titel: Physiological and Biochemical Characteristics of a New Group of Inotropic Agents: Analogues of Angiotensin II.

Previous Serial Number: None

Principal Investigator: Kenneth M. Kent, M. D., Ph. D.

Other Investigators: Theodore Goodfriend, M. D.
Theodore Cooper, M. D., Ph. D.

Cooperating Units: Department of Pharmacology, University of Wisconsin, Madison, Wisconsin

Project Description: Angiotensin II and several of its analogues have direct positive inotropic effects. In the course of investigating the effects of hypoxia on the activity of these agents we found that the inotropism of two of the angiotensin II analogues has a unique characteristic, i.e., potentiation by hypoxia. Thus, angiotensin II, des-1, val-5 produced a 50% increase in isometric contractile force in isolated cat papillary muscles at 2×10^{-7}M and a maximum increase in contractile force of 80% at 10^{-6}M; when the muscles were made hypoxic by equilibrating the Kreb's solution with an oxygen concentration of 5% instead of 95%, control tension decreased 32 $\pm$ 3% but the concentration-response curve for this compound shifted to the left and maximum response increased. The 50% increase in tension occurred at 5×10^{-9}M and the maximum increase in contractile force (at 10^{-6}M) was 190%. The responses to angiotensin, des-1, ile-5 were also greater in hypoxic papillary muscles. However, this compound was found to be less potent than the former.

The inotropic responses are independent of catecholamine stores since identical responses were obtained in papillary muscles from catecholamine-depleted muscles of chronically denervated cats. The potentiation produced by hypoxia is also independent of extracellular (Ca++). Present studies are being performed to determine the inotropic properties of these compounds when administered to animals with an intact coronary circulation.

In assessing the biochemical properties of these polypeptides, we have found that they bind specifically to submitochondrial phosphorylating particles (ETP_H) from beef heart. They increase the rate of phosphorylation, the tightness of coupling (respiratory control), and resistance to "aging" conditions of intact heart mitochondria. Phosphorylation rate is increased 40% by 5×10^{-8}g polypeptide/mg mitochondrial protein. Although the metabolic responses to these polypeptides improve oxidative metabolism, it is unclear whether this is the mechanism for their unique inotropic return in hypoxia.

Serial No. NHLI-14

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: Submitted for publication.

Serial No. NHLI-15
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Supersensitivity of Hyperthyroid Myocardium to Decreased Extracellular Calcium

Previous Serial Number: None

Principal Investigator: Kenneth M. Kent, M. D., Ph. D.

Other Investigators: Peter J. Dempsey, M. D.
Theodore Cooper, M. D., Ph. D.

Cooperating Units: None

Project Description: Previous workers have demonstrated that the contractile state of both the intact heart and isolated myocardial preparations obtained from hyperthyroid animals is increased. However, the cellular mechanisms responsible for the enhanced contractile state produced by thyroid hormone remains unknown. The purpose of this study was to determine the sensitivity of hyperthyroid myocardium to extracellular calcium concentration $[Ca^{++}]_e$. Two different processes presumed in part to reflect calcium transport in myocardial cells was measured: first, developed isometric tension (T) and rate of tension development (dT/dt); and second, the transmembrane action potential (TAP). At 0.45 mM $[Ca^{++}]_e$ T decreased 32 $\pm$ 3% in control and 51 $\pm$ 5% in hyperthyroid myocardium. At 0.15 mM $[Ca^{++}]_e$ the decrease in T was 54 $\pm$ 4% in control and 76 $\pm$ 3% in hyperthyroid muscles. Although the sensitivity of the contractile apparatus to $[Ca^{++}]_e$ of hyperthyroid myocardium was thus marked, the effect of Ca^{++} on TAP in hyperthyroid myocardium was similar to that in normal myocardium. These findings may provide some clue as to the mechanism of increased contractility of hyperthyroid myocardium.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Serial No. NHLI-16
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Control of Heart Rate in Hyperthyroid Cats

Previous Serial Number: None

Principal Investigator: Kenneth M. Kent, M. D., Ph. D.

Other Investigator: Theodore Cooper, M. D., Ph. D.

Cooperating Units: None

Project Description: The heart rates of anesthetized or alert hyperthyroid animals are increased, as are the intrinsic frequencies of contraction of hearts isolated from such animals. However, we have found that the basal heart rates of intact unanesthetized cats, in which the electrocardiogram was monitored by telemetry, were not different from those of normal cats. Basal heart rates were obtained by telemetry from the cats in the early morning hours while the cats remained in their cages. Any disturbance of the cats caused exaggerated increases in heart rates as compared to control animals. However, when the hearts were removed from these hyperthyroid cats and studied on a Langendorff apparatus isolated from neural or hormonal influences, the inherent sinus node rates were 40-50% higher than the rates of normal cats. These observations can be explained by the hypothesis that the negative chronotropic effect of the vagus is enhanced in hyperthyroid cats. This enhancement could have two mechanisms. First, the frequency of vagal nerve discharge could be increased, or second, the hyperthyroid hearts could be more sensitive to acetylcholine (Ach), the neurotransmitter. The latter hypothesis is being tested in isolated perfused hearts from hyperthyroid cats. Preliminary results have shown that several cardiac actions of Ach are enhanced in hearts from hyperthyroid cats. Thus, we have found that the direct negative inotropic effect of Ach, a muscarinic action, is enhanced in hyperthyroid myocardium, as is the release of norepinephrine from the ventricular myocardium, a nicotinic response to Ach. In addition, decreased conduction through the A-V node caused by Ach occurs at lower doses of Ach in hearts from hyperthyroid cats. Studies are in progress to determine the sensitivity of the sinus node to Ach in hearts from hyperthyroid cats.

Proposed Course of Project: Project continuing.

Honors and Awards: None

Publications: None

Serial No. NHLI-17
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Reports
July 1, 1970 through June 30, 1971

Project Title: Effects of Hypoxia on the Cardiac Response to Inotropic Interventions

Previous Serial Number: None

Principal Investigator: Kenneth M. Kent, M. D., Ph. D.

Other Investigators: Stephen E. Epstein, M. D.
Theodore Cooper, M. D., Ph. D.

Cooperating Units: None

Project Description: To evaluate the effects of hypoxia on the capacity of the heart to respond to various inotropic agents, cat papillary muscles were made hypoxic by changing the equilibrating gas mixture from 95% to 5% oxygen; pO_2 of the Krebs' solution decreased from 440 $\pm$ 8 to 122 $\pm$ 6 mm Hg. Baseline contractile force decreased 32 $\pm$ 3%, and the concentration-response curve of norepinephrine (NE) was depressed. Following the maximum response to NE, deterioration of developed tension occurred within 10 minutes. Maximum response to ouabain was less in hypoxia than under control conditions but no deterioration occurred after maximum response was achieved. The concentration-response curve of angiotensin II was unchanged under hypoxic conditions.

In order to determine if a similar hypoxia induced depression in inotropic activity occurred in intact hearts perfused with blood through their coronary arteries, a dog heart-lung preparation has been developed in which afterload and heart rate are held constant and preload is controlled by a resistance clamp. Left ventricular pressure, dp/dt, left and right coronary arterial flow and circuit flow are measured. Induction of hypoxia is accomplished by decreasing the inspired percentage of oxygen.

When arterial pO_2 was decreased from 100 to 20-25 mmHg (Hb saturation 40%) at a constant pH and pCO_2, there was an initial transient increase in maximum dp/dt. After return to control value, dp/dt remained stable for up to two hours. This stable hypoxic state was associated with an approximately four-fold increase in coronary flow. In this preparation, in contrast to cat papillary muscles, the contractile response to NE was augmented during hypoxia, the dose response curve shifted to the left. Furthermore, no deterioration of the preparation occurred after the highest doses of NE employed.

Studies are in the process to evaluate both the biochemical basis of this phenomenon and the effects of hypoxia on the response of this preparation to other inotropic agents.

Serial No. NHLI-17

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Serial No. NHLI-18(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1960 through June 30, 1971

Project Title: The Natural History of the Floppy Mitral Valve Syndrome

Previous Serial Number: None

Principal Investigator: Samuel B. Itscoitz, M. D.

Other Investigators: Stephen E. Epstein, M. D.
D. Luke Glancy, M. D.

Cooperating Units: None

Project Description: Until 1963, midsystolic clicks and late systolic murmurs were believed to be extracardiac in origin and of no clinical importance. In 1963, however, Barlow and co-workers first introduced evidence that these sounds were due to mitral valve disease with accompanying mitral regurgitation. This has been documented repeatedly by other investigators, and during the past eight years additional observations have been made. The disorder clusters in families and is probably congenital, although the murmur is not present at birth. It is related, in unclear fashion, to both Marfan's and Turner's syndromes. Anatomic material, when available, usually reveals a large redundant, slightly thickened posterior mitral valve leaflet with long thin chordae tendineae. Endocarditis has been reported and may result in chordae rupture in severe mitral regurgitation. ECG abnormalities are common and suggest inferior wall ischemia. Arrhythmias occur frequently, and sudden death has been reported.

Because of its rather recent recognition, however, the natural history of this disorder is not well known. Since the tension exerted on chordae tendineae is directly related not only to the intraventricular pressure but also to the area of the mitral valve leaflets to which it is attached, (i.e. the value leaflets act as an amplifier) a large redundant leaflet would cause greater than normal tension to be exerted on the chordae. Although one might thus anticipate that spontaneous chordal rupture would commonly complicate this abnormality, there is only one such case report in the literature at the present time.

In the past two years, two patients have been seen on the Cardiology ward who required mitral valve replacement for severe mitral regurgitation secondary to ruptured chordae tendineae. The ruptured chordae had attached to a billowing, redundant, floppy posterior mitral valve leaflet. Of interest, the typical auscultatory features of the floppy valve were not present in either of these patients; presumably the mechanism of regurgitation was no longer related to the floppy valve per se, but rather to the ruptured chordae. These additional cases provide further evidence that this complication may constitute

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a part of the natural history of this entity.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: In preparation

Serial No. NHLI-19(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Disseminated Intravascular Coagulation (DIVC) Complicating Severe Congestive Heart Failure (CHF)

Previous Serial Number: None

Principal Investigator: Samuel B. Itscoitz, M. D.

Other Investigators: Stephen E. Epstein, M. D.

Cooperating Units: None

Project Description: The syndrome of DIVC, an uncommon complication of a wide variety of disease states, has received increasing attention in the past 5 - 10 years. It is now commonly accepted that conditions in which thromboplastic substances gain access to the circulation (i.e., amniotic fluid embolism) or conditions in which regional blood flow is stagnant (i.e., shock of any origin) provide the milieu in which DIVC may occur. Nonetheless, reports of DIVC complicating severe CHF are very rare.

Despite its supposed rarity, in the past three years ten patients on the Cardiology ward have had relatively well documented episodes of DIVC. All patients were in severe congestive heart failure, usually biventricular in origin. The etiology of their heart disease was variable, including rheumatic heart disease, myocardiopathy, healed endocarditis, and idiopathic chordae tendineae rupture. Diagnosis of DIVC syndrome was based on the following laboratory studies: platelet count, presence of circulating fibrinogen degradation products, prothrombin time, thrombin time, partial thromboplastin time, factor V level, factor VIII level, and bleeding time.

Severity of the DIVC was quite variable, ranging from severe thrombocytopenia in one patient (who was found to have 70 gm. of fresh thrombus in the left atrium at operation) to mild transient thrombocytopenia in another. Clinical deterioration accompanied the DIVC episode in some, but not all, patients. Response to heparin, when administered, was variable. Evidence strongly suggestive of pulmonary embolic disease was commonly noted. One patient has been lost to followup. Of the seven known survivors, variable degrees of cardiac disability persist ranging from functional Class II to functional Class IV.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: In preparation.

Serial No. NHLI-20(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Radioisotope Imaging Technique for the Identification of Myocardial Scar

Previous Serial Number: None

Principal Investigator: Robert E. Goldstein, M. D.

Other Investigators: Douglas R. Rosing, M. D.
Gerald Schall, M. D.
Steven Larson, M. D.
Stephen E. Epstein, M. D.

Cooperating Units: Department of Nuclear Medicine
Clinical Center, N.I.H.

Project Description: The recent advent of direct surgical approaches to ischemic heart disease, utilizing either myocardial revascularization or resection of noncontracting portions of the ventricular wall, has made the distinction of viable contractile tissue from scar of crucial importance. At present, scarred regions of ventricular myocardium are identified as areas of abnormal wall motion on ventriculography. This technique, however, is indirect and insensitive. At the time of surgery the heart may be examined visually and surface electrograms obtained. But, aside from the necessity of thoracotomy, these methods also require that the epicardial layers are representative of the entire ventricular wall. Thus, a safe, sensitive radioisotope technique which would allow detailed mapping of living myocardial tissue in man without necessitating thoracotomy would be an important contribution to preoperative evaluation. The use of rubidium-84 in combination with the Auger positron camera configuration may represent such a technique.

Rubidium (Rb) is an alkali metal whose chemical and biological behavior closely mimics that of potassium. When administered intravenously Rb accumulates rapidly in heart and skeletal muscle (and other organs) so that it is almost entirely removed from the blood stream within two minutes. This property has been utilized to measure coronary flow in man. After intravenous or intracoronary administration of the positron-emitting isotope Rb-84, a coincidence counting scintillation camera is used to identify those areas of the myocardium which fail to take up Rb-84 due to replacement of muscle tissue by scar. This technique has reportedly been successful in localizing areas of infarction in dogs. Coincidence counting should provide a high resolution image, theoretically resolving 0.5 cm points apart. Positron emission also allows "focusing" of the camera so that its tomographic "cuts" of the chest can be obtained. Thus, a three-dimensional array of radioactivity can be

mapped and "focused" further, using a digital computer. Preliminary studies with open-chest dog preparations have demonstrated the ability of Rb-84 to delineate the myocardium even on intravenous injection. Intravenous administration of Rb-84 in two patients, however, failed to permit adequate imaging of the myocardium. We currently plan to determine whether intracoronary administration will result in sufficient myocardial concentration to result in a satisfactory scan. We also plan to evaluate the imaging properties of potassium-43, an isotope reported to be useful as a myocardial scanning agent.

Proposed Course of Project: Project continuing.

Honors and Awards: None

Publications: None

Serial No. NHLI-21
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Correlation of Antiarrhythmic Effects of Diphenylhydantoin with Digoxin-induced Changes in Contractility, Na-K ATPase and K^+ Efflux

Previous Serial Number: None

Principal Investigator: Robert E. Goldstein, M.D.

Other Investigators: S. C. Penzotti, M.D.
Karen S. Kuehl, M.D.
Kirk H. Prindle, M.D.
Clifford A. Hall, M.D.
Elwood O. Titus, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Laboratory of Chemical Pharmacology, NHLI

Project Description: To clarify the suppressant action of diphenylhydantoin on digitalis-induced arrhythmias we studied the effects of diphenylhydantoin and digoxin, alone and in combination, on contractile force, Na-K ATPase activity and K^+ efflux in Krebs-Ringer perfused dog hearts. Neither control perfusion nor diphenylhydantoin alone (3×10^{-5}M) altered Na-K ATPase or produced K^+ efflux. Diphenylhydantoin alone depressed contractile force an average of 29% at 15 min. Digoxin alone (10^{-6}M) in 7 dogs caused a 59% rise in contractile force at onset of arrhythmia (toxicity) (avg. 15.9 min of perfusion $\pm$ 0.7 SE) along with net K^+ efflux of 50 $\pm$ 12 μM/min and decrease in Na-K ATPase from 13.8 to 5.2 μM Phos/mg protein/hr. ($P < 0.001$). Perfusion with diphenylhydantoin and digoxin combined (5 dogs) delayed toxicity to 28.9 $\pm$ 2.8 min, at which time contractile force was higher, 91% above control ($P < 0.05$), K^+ efflux tended to be greater (87 $\pm$ 20 μM/min) and Na-K ATPase was lower (2.5, $P < 0.05$) than with digoxin alone. Combined diphenylhydantoin-digoxin perfusion lasting only until the time toxicity appeared with digoxin alone (7 dogs), however, yielded higher Na-K ATPase (7.7, $P < 0.02$) compared with digoxin alone; K^+ efflux was unchanged and contractile force decreased (26% above control, $P < 0.05$). Thus diphenylhydantoin appears to diminish the rate at which digoxin inhibits Na-K ATPase. Nevertheless, diphenylhydantoin ultimately permits digoxin to produce greater inhibition of Na-K ATPase, greater increase in contractile force, and a tendency toward greater K^+ efflux than possible without diphenylhydantoin. This suggests that the antiarrhythmic effects of diphenylhydantoin cannot be attributed to prevention of inhibition of Na-K ATPase or to diminution of K^+ efflux, two changes characteristically accompanying digoxin administration.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript in preparation

Serial No. NHLI-22
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hemodynamic Changes During Exertional Syncope in Dogs with Pulmonary Artery Constriction

Previous Serial Number: None

Principal Investigator: Robert E. Goldstein, M. D.

Other Investigators: Alan Nimetz, M. D.
Joseph Pierce, D. V. M.
Stephen E. Epstein, M. D.

Cooperating Units: None

Project Description: Individuals with severe obstruction to outflow of either the right or left ventricles may experience syncope during or immediately after strenuous exercise. In order to clarify the hemodynamic basis for this phenomenon, several circulatory indices were measured while dogs with pulmonary artery constriction exercised to the point of syncope. Pulmonary constriction was achieved by the inflation of a Jacobsen balloon cuff previously sewn around the pulmonary artery. During the course of treadmill exercise, continuous recordings were made of systemic arterial, right ventricular, and distal pulmonary artery pressures and the electrocardiogram. In several animals aortic blood flow was measured by a circumferential electromagnetic flowmeter cuff. Preliminary results showed that syncope was almost always accompanied by rapidly progressive arterial hypotension rather than by arrhythmia. Decline in cardiac output generally accompanied the fall in blood pressure, suggesting that inadequacy of cardiac output rather than sudden vasodilatation was responsible for the decline in blood pressure which led to circulatory collapse. Further experimental studies will be performed in animals with implanted flowmeters in order to substantiate these preliminary findings.

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

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Serial No. NHLI-23
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Comparison of Simultaneously Determined Inotropic and Chronotropic Effects of Practolol and Propranolol

Previous Serial Number: None

Principal Investigator: Robert E. Goldstein, M.D.

Other Investigators: Clifford A. Hall, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Recent reports have differed concerning the relative negative inotropic effects of the β-blockers practolol and propranolol. To resolve these conflicting findings, simultaneous changes in contractile force and heart rate were measured when increasing doses of either propranolol or practolol were given to dogs receiving infusions of isoproterenol. Infusion was adjusted initially to produce a mean heart rate of 155; blood pressure was held constant. Contractile force was measured by a right ventricular strain gauge arch. When drug dosages producing equal decreases in heart rate were compared, the associated decrement in contractile force was not significantly different; for example, when heart rate decreased 15%, contractile force was lowered 37% with practolol and 41% with propranolol (NS). Thus, a given reduction in heart rate was accompanied by the same negative inotropic response with both drugs. Effective β-blocking doses of propranolol (up to 1 mg/Kg) given after β-blockade had been produced by large doses of practolol caused no further depression in contractile force. These findings suggest that depression by propranolol in doses less than 1 mg/Kg is largely related to β-blockade; primary depressant effects were seen only with doses above 1 mg/Kg. Practolol after large doses of propranolol, however, increased contractile force and heart rate: at 2-5 mg/Kg contractile force increased 33% and heart rate 6%. Thus practolol may exert a modest positive inotropic effect detectable after full β-blockade or possibly when β-receptor stimulation is minimal. Nevertheless, such an effect is inapparent if β-receptor stimulation is substantial, which is often the case when β-blockade is employed clinically. Thus, practolol as used therapeutically, does not appear to act on heart rate more selectively than propranolol.

Proposed Course of Project: Continuing

Honors and Awards: None

Publications: None

Serial No. NHLI-24(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Chronic Heart Failure on the Capacity of Glucagon to Enhance Contractility and Adenyl Cyclase Activity of Human Papillary Muscles

Previous Serial Number: None

Principal Investigator: Robert E. Goldstein, M.D.

Other Investigators: C. Lynn Skelton, M.D.
Gerald S. Levey, M.D.
D. Luke Glancy, M.D.
G. David Beiser, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Glucagon exerts a positive inotropic effect in normal hearts but is ineffective in animals with chronic cardiac failure. To assess directly the influence of glucagon on human myocardium, we measured contractility and activation of adenyl cyclase, the enzyme thought to mediate the inotropic action of glucagon, in left ventricular papillary muscles obtained from 12 patients at mitral valve replacement. On the basis of preoperative ventricular end-diastolic pressures and cardiac output (independent of papillary muscle data) patients were classified in 3 groups: normal, with cardiac failure, or indeterminate. Concentration-response curves showed that glucagon caused a mean 11% rise in peak papillary muscle tension and 12% rise in peak rate of tension development in the normal patients; myocardial adenyl cyclase activity from each patient rose after glucagon (average = 8%). In the papillary muscles of the patients with cardiac failure, glucagon produced no augmentation in either tension or adenyl cyclase activity. In contrast, contractility and adenyl cyclase activity increased after norepinephrine in both normal patients and those with cardiac failure. The indeterminate group had two patients whose papillary muscles responded to glucagon and two whose papillary muscles did not respond. Thus, direct study of human papillary muscles shows that chronic cardiac failure is uniformly associated with a complete loss of the normal enhancement of contractility and associated activation of adenyl cyclase after glucagon, perhaps explaining the inefficacy of this drug in treating patients with chronic cardiac failure.

Proposed Course: Completed

Honors and Awards: None

Publications: Manuscript submitted for publication.

Serial No. NHLI-25
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Comparison of the Inotropic and Chronotropic Activity of Glucagon and Isoproterenol in the Intact Canine Heart

Previous Serial Number: None

Principal Investigator: Robert E. Goldstein, M.D.

Other Investigators: David R. Redwood, M.D.
G. David Beiser, M.D.
Alan Nimetz, M.D.
Joseph Pierce, D.V.M.
Stephen E. Epstein, M.D.

Cooperating Units: Division of Research Resources, Bureau of Health Professions Education & Manpower Training
Laboratory of Kidney and Electrolyte Metabolism, National Heart and Lung Institute

Project Description: Although glucagon and isoproterenol are both known to be powerful inotropic agents, the relative potency of these two drugs has not been systematically assessed. We therefore compared the cardiac effects of glucagon and isoproterenol in each of 10 dogs. Using a strain gauge sewn to the right ventricle, dose-response curves were obtained for each drug while arterial pressure was held constant. Cardiac output was measured at the peak dose (maximal strain gauge deflection) of each drug. Results were similar whether glucagon was infused continuously or given as a bolus. Peak doses of both glucagon and isoproterenol increased strain gauge deflection from a mean of 15 to 36 mm (paired comparison of strain gauge deflections at peak glucagon and isoproterenol showed a mean difference of 0.24 ± 2.0 mm SE). Heart rate at peak dose averaged 217 beats/min for glucagon and 195 beats/min for isoproterenol ($P < .005$). Cardiac output averaged 4.9 L/min during peak glucagon and 6.6 during peak isoproterenol ($P < .05$). In conclusion, direct comparison of glucagon and isoproterenol in the normal canine heart showed the maximal inotropic effect of glucagon to be indistinguishable from that of isoproterenol. In contrast to previous impressions, peak doses of glucagon produced marked tachycardia, exceeding that of peak isoproterenol when pressure was held constant. Under these same circumstances peak glucagon also increased cardiac output considerably, although less than peak isoproterenol. Studies in three dogs with chronic congestive heart failure due to pulmonary artery constriction showed a marked reduction in glucagon responsiveness consistent with diminished glucagon responsiveness observed in chronically failing myocardium of cat and man.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: None

Serial No. NHLI-26(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Dynamic Nature of Left Ventricular Outflow Obstruction in Idiopathic Hypertrophic Subaortic Stenosis

Previous Serial Number: None

Principal Investigator: D. Luke Glancy, M. D.

Other Investigators: Richard L. Shepherd, M. D.
G. David Beiser, M. D.
Stephen E. Epstein, M. D.

Cooperating Units: None

Project Description: Left ventricular outflow obstruction in idiopathic hypertrophic subaortic stenosis (IHSS) is caused by midsystolic apposition of the disproportionately hypertrophied cephalad portion of the ventricular septum with the free margin of the anterior mitral valvular leaflet, and the degree of obstruction is variable. Unusual findings during hemodynamic studies in three patients support the hypothesis that the degree of obstruction in this condition is determined by three factors: the force of ventricular contraction, ventricular volume, and ventricular afterload. In one patient spontaneous atrioventricular dissociation resulted in spontaneous changes in left ventricular end-diastolic pressure, and presumably in end-diastolic volume. With increases in end-diastolic pressure and volume the degree of left ventricular outflow obstruction decreased, whereas with decreases in end-diastolic pressure and volume the degree of obstruction increased. During left ventricular pulsus alternans in this same patient, the more forceful ventricular contractions resulted in an increase in the degree of obstruction, and peak pressure in the systemic arteries did not alternate.

In the second patient PVC responses, which initially were typical of IHSS, transiently became normal following the injection of angiographic contrast material, suggesting that an increase in ventricular volume and depression of myocardial contractility temporarily relieved the left ventricular outflow obstruction. In the third patient a spontaneous increase in systemic arterial pressure, and thus in left ventricular afterload, completely abolished a 100 mm Hg subaortic systolic gradient.

Proposed Course of Project: Completed.

Honors and Awards: None

Publications: Submitted for publication.

Serial No. NHLI-27
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Dissociation of Cardiac Inotropic and Membrane Effects of Ouabain

Previous Serial Number: NHLI - 21

Principal Investigator: Peter J. Dempsey, M. D.

Other Investigators: Kenneth M. Kent, M. D., Ph. D.
Theodore Cooper, M. D., Ph. D.

Cooperating Units: None

Project Description: Ouabain causes negative inotropic responses in cat papillary muscles when the external calcium concentration, $[Ca^{++}]_e$, is 0.45 mM or lower. This negative inotropism occurs with shortening of phase 2 of the transmembrane action potential (TAP), the latter being the characteristic response to ouabain.

Further studies have shown that the negative inotropism was dose dependent. Ouabain 4×10^{-7}M at $[Ca^{++}]_e$ = 0.15 mM produced at 12 $\pm$ 1% decrease in isometric contractile force whereas ouabain 3×10^{-7}M caused a 41 $\pm$ 6% decrease. Norepinephrine, isoproterenol, and angiotensin II produced their usual inotropic responses independent of the $[Ca^{++}]_e$.

When ouabain was added to cat papillary muscles at $[Ca^{++}]_e$ lower than 0.45 mM, there was an initial lengthening of phase 2 of the TAP. This was a transient response for as the negative inotropic response to ouabain became established, phase 2 shortened in the characteristic manner for ouabain.

A possible explanation for these events is a ouabain-induced loss of Ca^{++} from the cell into the perfusing solution which has a low $[Ca^{++}]_e$. This loss of Ca^{++} would explain the lengthening of the TAP and the negative inotropic response.

Proposed Course of Project: Completed

Honors and Awards: None

Publications:

ARTICLE PUBLISHED IN PERIODICAL:

Dempsey, P.J., McCallum, Z.T., Kent, K.M., and Cooper, T.: Dissociation of cardiac inotropic and membrane effects of ouabain. J. Pharm. Exp. Therap. 176: 78 , 1971.

Serial No. NHLI-28
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Calcium Ion Movement in Skeletal Muscle

Previous Serial Number: None

Principal Investigator: Chester E. Clark, M. D.

Other Investigators: None

Cooperating Units: None

Project Description: Current theory regarding excitation-contraction coupling in cardiac and skeletal muscle centers around the concept that calcium, released by the sarcoplasmic reticulum (SR) following electrical depolarization, diffuses from SR to a receptor located on the actin filament. Then, through a series of as yet unknown transformations, Ca is believed, in this location, to cause the interaction of actin and myosin filaments, thereby initiating muscle shortening. Implicit in this hypothesis is the assumption that the rate of diffusion of Ca is sufficient to account for the rapid sequences of contraction and relaxation characteristic of cardiac and skeletal muscle. However, the only data relating to the rate of calcium ion movement in muscle cytoplasm indicate that mobility is at least 50 to 100 times slower than in water, a rate probably too slow to be compatible with the above hypothesis. If the slow rate measured in muscle cytoplasm can be attributed to uptake by the SR, then faster rates would be expected in muscle subcompartments where SR envelops the contractile apparatus and is structured to produce minimal interference with calcium mobility. It would also be expected that electrical depolarization would minimize calcium uptake by SR.

In order to ascertain if the intracellular movement of calcium between subcellular compartments is rapid enough to control contraction of striated muscle, an analogue circuit for the solution of diffusion problems was constructed. Using the known binding constants for SR it was found that uptake of calcium by SR could not account for the slow movements of calcium intracellularly. This finding thus raises the possibility that the classical hypothesis for excitation-contraction coupling may be in error.

To confirm this finding experimentally in living tissue, individual myofibrils were isolated by homogenation and centrifugation, and the measurements of the delay in onset of contraction after exposure to calcium was attempted by measuring alterations in optical density. This technique was employed since it has been reported that optical density changes in parallel to the degree of myofibrillar contraction. However, no correlation was discovered between myofibrillar contraction and optical density, a finding calling into

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question many studies published in the literature based on such methodology.

Two other approaches of measuring Ca diffusion rates in vivo are being developed. First, we are in the process of developing techniques to isolate single muscle fibers from the frog semitendinosus muscle. The fiber will be cut across one end and exposed to calcium^{45}. After varying periods of time the muscle will be sliced and the distribution of calcium^{45} determined. From this data a diffusion rate can be calculated.

Finally, we are trying to utilize high speed photomicrography to detect the delay of myofibrillar contraction after exposure to calcium.

Proposed Course of Project: Project continuing.

Honors and Awards: None

Publications: None

Serial No. NHLI-29
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Treatment of Ventricular Arrhythmias Occurring During Acute Coronary Artery Occlusion in Conscious Dogs: Efficacy of Atropine and Lidocaine

Previous Serial Number: None

Principal Investigator: G. David Beiser, M.D.

Other Investigators: Douglas R. Rosing, M.D.
Richard B. Karsh, M.D.
James Talano, M.D.
Martin Miller
James Bailey, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: Division of Computer Research and Technology
(Computer Systems Laboratory and Laboratory of Applied Studies)

Project Description: Ventricular arrhythmias associated with acute myocardial infarction may seriously compromise cardiac output or result in a fatal episode of ventricular fibrillation or cardiac standstill. In order to evaluate the treatment of such arrhythmias, we studied 40 closed-chest conscious dogs in which acute myocardial ischemia was produced by inflating a balloon cuff previously implanted around the left anterior descending coronary artery just distal to its first diagonal branch. Of the 40 dogs, 15 developed frequent and persistent ventricular ectopic beats and/or episodes of ventricular tachycardia within the first one or two hours of occlusion. In 5 dogs the arrhythmia progressed rapidly to ventricular fibrillation and death. In the remaining 10 dogs increasing the heart rate with the intravenous administration of atropine markedly decreased or completely abolished the arrhythmia. However, in some of the dogs it was necessary to increase the heart rate to as high as 140 - 160/min to suppress the arrhythmia. The intravenous administration of lidocaine in a dose of 2 mg/kg either decreased the number of premature beats or abolished the arrhythmia completely. In three dogs the administration of atropine to a heart rate of 120/min, or lidocaine at a dose of 2 mg/Kg, were only partially effective in the treatment of the arrhythmia. However, the combination of these two drugs eliminated all ectopic activity.

Proposed Course of Project: Additional dogs will be studied to further define the efficacy of atropine and xylocaine, alone and in combination, in the treatment of arrhythmias associated with acute experimental myocardial infarction.

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Honors and Awards: None

Publications: None

Serial No. NHLI-30(c)
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effects of Operative Correction of Congenital Heart Disease on the Functional Capacity of the Heart

Previous Serial Number: NHLI-34(c)

Principal Investigator: G. David Beiser, M. D.

Other Investigators: Stephen E. Epstein, M. D.
Robert E. Goldstein, M. D.
Douglas R. Rosing, M. D.
David R. Redwood, M. D.
Andrew G. Morrow, M. D.

Cooperating Units: Clinic of Surgery, NHLI

Project Description: In the past decade great strides have been made in the operative treatment of patients with congenital heart disease. However, progress in the methods used to evaluate the functional results of such operations have lagged far behind. Thus, while we know that many patients with severe cyanotic congenital heart disease experience marked symptomatic improvement after operative correction, we have little information as to whether the functional capacity of the heart is completely normal. We also have no information relating to the effects on cardiac function of a ventriculotomy or of the insertion of a patch in the right ventricular outflow tract. With the recent development in this laboratory of a sensitive technique to evaluate the maximal pumping capacity of the heart, a study was undertaken with the purpose of answering these questions. In brief, the cardiac output, oxygen uptake, and right ventricular and pulmonary arterial pressures were determined during maximal treadmill exercise in patients in whom an atrial septal defect or tetralogy of Fallot had been repaired six months to three years previously.

Seven of eleven asymptomatic patients with closed atrial septal defects had a significant impairment in their cardiac output response to exercise. In addition, one of three patients who had pulmonary arterial hypertension preoperatively developed abnormally high pulmonary pressures during intense exercise. Of the ten patients with correction of tetralogy of Fallot who have been studied, eight showed a small but definite impairment in the pumping capacity of the heart; two had a normal cardiac output at intense exercise. Although it might have been expected that patients with tetralogy of Fallot would have a reduced capacity of the pulmonary vascular bed and thus develop pulmonary hypertension during maximal exercise, none of the ten patients developed abnormal pulmonary arterial pressures.

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However, right ventricular systolic pressure, measured in six patients, increased in four from normal or minimally-elevated levels at rest to greater than 85 mmHg during intense exercise. This elevation was due to increases in right ventricular outflow gradients ranging from 50 to 65 mmHg. Finally, the presence of significant pulmonic regurgitation in five patients with corrected tetralogy of Fallot did not appear to compromise their cardiac performance relative to the other patients.

Proposed Course of Project: Completed

Honors and Awards: None

Publications: Manuscript in preparation.

Serial No. NHLI-31
1. Cardiology
2. Clinical Physiology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ventricular Tachycardia Following Release of Coronary Artery Occlusion in Conscious Dogs and the Antiarrhythmic Effects of Atropine

Previous Serial Number: None

Principal Investigator: G. David Beiser, M.D.

Other Investigators: Douglas R. Rosing, M.D.
Richard B. Karsh, M.D.
James Talano, M.D.
Stephen E. Epstein, M.D.

Cooperating Units: None

Project Description: Arrhythmias occurring in the acute stages of myocardial infarction generally have been attributed to persistent myocardial ischemia secondary to coronary artery occlusion. To evaluate the mechanisms and treatment of such arrhythmias, we studied 40 closed-chest conscious dogs in which acute myocardial ischemia was produced by inflating a balloon cuff previously implanted around the left anterior descending coronary artery just distal to its first diagonal branch. Of the 40 dogs, 18 did not develop arrhythmias during the first hour of occlusion. In 11 of these 18, however, sudden release of occlusion was followed 15 seconds to 3 minutes later by ventricular arrhythmias which progressed rapidly to ventricular tachycardia. In all 11 dogs reocclusion of the coronary artery abolished the ventricular tachycardia within 30 seconds with a return to the sinus mechanism. Reocclusion for 10 to 15 minutes followed by successive periods of release and reocclusion consistently reproduced this phenomena. In 5 of 6 dogs atrial pacing at rates of 105 to 130 beats per minute was successful in over-riding release-induced ventricular tachycardia or preventing its appearance. Likewise, the administration of atropine in 10 dogs just prior to release of occlusion in doses that increased heart rate from an average of 76 $\pm$ 6 (standard error of the mean) to 112 $\pm$ 3 beats per minute prevented ventricular tachycardia in all dogs.

Release arrhythmias were also studied in an additional group of 5 dogs in which transient ventricular arrhythmias occurred during the first hour of occlusion. Release of occlusion in these animals resulted in very malignant appearing ventricular tachycardias which on the first or subsequent releases progressed rapidly to ventricular fibrillation in 3 dogs despite therapeutic attempts.

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These results indicate that 1) sudden reperfusion of a previously ischemic region of myocardium, as may occur in man by lysis or dislodgment of a clot, may be responsible for some of the serious arrhythmias seen in acute myocardial infarction which result in sudden death; 2) increasing the heart rate by the administration of atropine may be successful in the prevention of many but not all of these arrhythmias.

Proposed Course of Project: To further define the character of these arrhythmias in relation to the onset of occlusion and to study the effects of other therapeutic agents in preventing their appearance with release of occlusion.

Honors and Awards: None

Publications: None

Serial No. NHLI-32
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanical behavior of large coronary arteries.

Previous Serial Number: NHLI-47

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: Thomas E. Carew, Ph.D.

Project Description:

Objective: To study 1) the rheology and 2) the effect of increased stress on the protein permeability of the endothelial surface of the left circumflex coronary artery (LCCA) and the anterior descending coronary artery (ADCA).

Methods Employed: 1) The study evaluating the static elastic properties of LCCA has been completed (see publication). 2) Left coronary artery was perfused in situ at various perfusion pressures and flows with oxygenated blood containing known amounts of Evans blue dye for approximately 1 hour. At the end of the experiment the endothelial surface of both LCCA and ADCA was exposed and examined under a reflectometer for blue staining. The permeability of the endothelium to albumin was evaluated by quantifying the blue staining of the endothelial surface. The amount of dye bound albumin that had entered the surface was correlated with the shearing stress as well as the pressure to which the artery was subjected during the perfusion experiment.

Major Findings: The data was grouped into 4 groups: 1) High shearing stress (average value 30 dynes/cm^2) and high pressure (average value 170 cm H_2O); 2) high shearing stress and low pressure; 3) low shearing stress and high pressure and 4) low shearing stress and low pressure. Preliminary results suggest that high shearing stress led to a greater dye concentration in the intima than did high pressure.

Significance to Bio-medical Research and the Program of the Institute: Although the importance of hydrodynamic and rheologic factors in early vessel damage has been recognized, its role has not been critically evaluated in the coronary vascular bed to date. The present studies are designed toward better understanding of the role of mechanical stress in producing vascular diseases.

Proposed Course of the Project: 1) Studies to evaluate the dynamic visco-elastic properties of the LCCA are in progress. 2) Studies are in progress a) to delineate the role of shearing stress vs normal stress in increasing the permeability of the endothelial surface, and b) to define the critical stress at which the endothelium deteriorates.

Honors and Awards: None.

Serial No. NHLI-32

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Publications:

Patel, D.J., and Janicki, J.S.: Static elastic properties of the left coronary circumflex artery and the common carotid artery in dogs. Circulation Res. 27: 149-158, 1970.

Serial No. NHLI-33
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Blood velocity profiles and wall shear in the aorta and its major branches.

Previous Serial Number: NHLI-48

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: Donald L. Fry, M.D.
Joseph S. Janicki, M.S.
S. C. Ling, Ph.D.
H. Bulent Atabek, Ph.D.

Cooperating Units: The Department of Space Science and Applied Physics, The Catholic University of America

Project Description:

Objective: To measure and quantify 1) the blood velocity fields, 2) turbulence, and 3) the interfacial shearing stress on the endothelial surface along the major arteries of dogs through the cardiac cycle under various conditions.

Methods Employed: A constant-temperature heated film anemometer system has been adapted for direct measurement of in vivo aortic velocity fields. Although the method is promising all technical problems are not yet resolved and effort in the past year was directed towards these. Also progress is made towards automatic data processing without which considerable time would be spent in plotting velocity fields from sequentially obtained velocity curves within the cross-section of the aorta.

Major Findings: Preliminary results indicate that velocity profiles along the descending thoracic aorta are essentially blunt; slight skewing is observed which may be due to flow being diverted to intercostal arteries.

Significance to Bio-medical Research and the Program of the Institute: The method provides a powerful tool in quantitative investigations of the vascular system. It will help assess the role of hydrodynamic factors in potentiating the development of atheroma in the major arteries.

Proposed Course of the Project: A numerical technique has been developed and verified in models at Catholic University to compute velocity profiles and wall shearing stress from measurement of pressure, pressure gradient and radius in a straight segment of a tube. Attempts will be made to test this indirect but easier method vs direct measurements of wall stress and velocity profiles in a dog.

Serial No. NHLI-33
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Honors and Awards: None.

Publications: None.

Serial No. NHLI-34
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Vascular mechanics: arterial wall properties

Previous Serial Number: NHLI-49

Principal Investigator: Dali J. Patel, M.D., Ph.D.

Other Investigators: Joseph S. Janicki, M.S., R. N. Vaishnav, Ph.D.

Cooperating Units: Department of Civil and Mechanical Engineering, The Catholic University of America, Washington, D. C.

Project Description:

Objective: To study the nonlinear, visco-elastic properties of the blood vessel under physiologic conditions with special emphasis on measurement of "strain energy density."

Methods Employed: A segment of the middle descending thoracic aorta was studied in dogs. The pressure-radius-longitudinal force relationship was studied (using specially designed transducers) by connecting the segment to a reservoir filled with oxygenated blood and adjusting the height of the reservoir to cover the physiologic range of pressure. In addition, a flow pump was connected to this assembly to superimpose sinusoidal pressures at various frequencies on the segment to study its dynamic properties. From these data it was possible to compute 1) the values of elastic constants to describe the non-linear behavior of the vessel wall and 2) the incremental visco-elastic constants in the three principal directions (radial, circumferential and longitudinal).

Major Findings: 1) Previous studies in the laboratory have shown that the blood vessel wall is essentially incompressible and demonstrates anisotropic behavior with a certain elastic symmetry. 2) The values of incremental visco-elastic coefficients in the three principal directions increased with frequency. 3) The values of the elastic part of these coefficients (real part of the complex coefficient) were much greater than their viscous counterpart. 4) The nonlinear elastic behavior of the vessel wall could be described adequately in the physiologic range of pressures by 7 constants. From these data it was possible to predict the values of incremental elastic constants as well as strain energy density at any desired value of pressure. This latter finding was extremely useful for precise comparison of data among different animals.

Significance to Bio-medical research and the Program of the Institute: Detailed experimental studies like this are essential for building a mathematical theory to describe the vascular system which in turn would complement our Section's program on experimental atherosclerosis. The reasons for this are twofold: 1) The dynamic behavior of the vascular

system depends critically upon the physical properties of the vessel wall and its tethering mechanism. These properties had not been adequately quantified prior to these studies. 2) Recent studies in our laboratory have indicated that an increased strain energy density in the vessel wall may act as a driving force to increase the flux of lipoproteins across the vessel wall. Thus, it becomes important to establish the nature and magnitudes of the vessel wall elastic moduli so that the strain energy density of the wall can be calculated and compared to the distribution of early experimental atheroma.

Proposed Course of the Project: The studies will be continued 1) to include in vivo dynamic anisotropic properties of the blood vessel wall; and 2) to provide an experimental basis for a nonlinear visco-elastic theory of the blood vessel wall.

Honors and Awards: None

Publications:

Patel, D.J., and Vaishnav, R.N.: Rheology of large blood vessels. Cardiovascular Fluid Dynamics. Academic Press, Inc. London. In press.

Serial No. NHLI-35

1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In vitro studies of the influence of mechanical factors on transvascular albumin flux.

Previous Serial Numbers: None

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objective: To study the effect of various mechanical factors on the flux of albumin across the vascular interface.

Methods Employed: Freshly removed canine aortic tissue was placed in a specially designed device (described in previous reports) such that the interface could be exposed to varying predetermined degrees of stretch and trnasmural pressure, while being covered with autologous serum containing Evans blue tagged albumin. Intact endothelial surfaces, as well as surfaces that had been denuded by gentle stroking with a camel's hair brush were studied.

Major Findings: It was found that the flux of albumin across the vascular interface was increased by stretching the surface. In contrast to this transmural pressure drops up to 280 cm of water pressure caused no change in the flux across the surface, either in the intact preparation or in that in which the endothelial cell surface had been removed. The removal of endothelial cells increases the permeability of the vascular interface by at least 2 orders of magnitude.

Significance to Bio-medical Research and the Program of the Institute: These studies shed light on the mechanical factors which influence the transport of albumin across the vascular interface. They indicate that the greatest barrier to protein flux resides in a small region associated with the endothelial cell layer. The fact that pressure does not influence the flux of albumin into the wall, either in the presence of a normal barrier, or in its absence, suggests that the arterial wall buttresses the endothelial cells and their associated barrier against the forces of pressure rendering pressure ineffective as a driving force for protein transport. Mechanical events analogous to the foregoing may also be operative in the vascular system and of significance in the transport of β-lipoprotein in the pathogenesis of atherosclerosis.

Proposed Course of the Project: Studies, such as the above, will be repeated with various other proteins and, in particular, with β-lipoprotein when appropriate tagging techniques become available for their quantification.

Serial No. NHLI-35
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Honors and Awards: None

Publications:

Fry, D.L.: Localizing factors in arteriosclerosis. Ch. II. Atherosclerosis and Coronary Heart Disease. Hahnemann Symposium, 1971. In press.

Serial No. NHLI-36
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In vitro study of the influence of temperature on transvascular albumin flux.

Previous Serial Numbers: None

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objective: To quantify the effective temperature on protein flux into the vascular wall.

Methods Employed: Freshly removed canine aortas were placed in a specially designed device such that various portions of the vascular segment could be held at different prescribed temperatures. Each of these areas was then exposed to autologous serum containing Evans blue tagged-albumin for specified periods of time and the subsequent flux of albumin into the wall measured.

Major Findings: Below 300° K the flux of albumin into the vascular interface appears to follow an exponential relationship suggesting that it could be an activated diffusion process. Above 312° K the resistance of the diffusion barrier virtually disappears resulting in massive influx of protein into the wall. Histologic studies suggest that the endothelial cells below this temperature remain normal and above this temperature suffer changes in staining properties and become pyknotic.

Significance to Bio-medical Research and the Program of the Institute: There has been evidence from this laboratory suggesting that the permeability of the vascular interface may be related to its internal energy density. If so, it would be expected also to show the characteristic temperature-flux relationship of an activated diffusion process. While the foregoing data are of a preliminary nature and as yet show too much scatter to prove the hypothesis, they are nevertheless suggestive. The data also suggest that at temperatures in excess of 300 K the vascular interface appears suddenly to "melt," which is consistent with the idea that part of the barrier may reside in the cell membranes which are known to have a transition in this range.

Proposed Course of the Project: Further studies will be done to refine the measuring techniques so that the functional relationships between temperature and flux can be quantified more accurately. Electron microscopic and histochemical studies of the tissue will be done in an

effort to identify changes in composition of the wall with temperature, particularly those associated with the "melting" process. Finally, these studies will be repeated with various lipoproteins when suitable tagging techniques for their quantification become available.

Honors and Awards: None

Publications: None

Serial No. NHLI-37
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: In vivo studies of aortic intimal histologic and chemical response to acutely induced mechanical stress.

Previous Serial Numbers: NHLI-41

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: Section on Pathology, Cardiology Branch

Project Description:

Objective: To continue in vivo studies of the intimal histologic and chemical response to acutely induced mechanical stress in shear, tension, and compression.

Methods Employed: A variety of mechanical devices have been devised which allow controlled mechanical stress to be applied to the vascular intima in a variety of configurations in the acute animal preparation. The details of these methods have been described in previous reports. Additional methodology was developed to quantify the tissue response to the above induced stresses. Two new techniques were developed for estimating the flux of Evans blue tagged albumin across the vascular interface. The first was a direct reflectometric approach in which the freshly opened stretched vascular interface is mounted in an evenly illuminated thermally and chemically controlled bath. The image of the submerged interface is projected by a lens system to a large ground glass plate, one point of which is scanned by a photocell. With an appropriate selection of filters it is possible to measure the amount of light absorbed in the blue-stained regions as a function of the Evans blue concentration in the vessel surface. The second method is a fluorescence technique based on an observation which we have made that Evans blue bound albumin fluoresces at about 630 nanometers when excited by the green band of the mercury spectrum. The amount of red light emitted can then be quantified using appropriate filter systems such that the concentration of Evans blue may be related to the fluorescence intensity.

Major Findings: The above studies have corroborated the results of previous studies in this laboratory in that the vascular interface has been found to be responsive to applied mechanical stress: endothelial cells yield at about 400 dynes/cm^2 and the permeability of the vascular interface to protein increases at stresses below this value. The reflectometric measurement of staining density distribution of Evans blue dye on the opened vascular interface was found to correlate well with the intensity of Evans blue albumin fluorescence elicited from the corresponding histologic section.

Serial No. NHLI-37

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Significance to Bio-medical Research and the Program of the Institute:
Both experimental as well as naturally-occurring atherosclerosis is a discrete process occurring in the vascular intima, the topography of which suggests that the development of atherosclerotic lesions tends to be accelerated in regions exposed to increased mechanical stress. If the mechanism of this process is to be studied, then it is essential to develop techniques for studying discretely the transport mechanics of the vascular interface.

Proposed Course of the Project: This project has been completed and data is being analyzed and prepared for publication.

Honors and Awards: None

Publications: None

Serial No. NHLI-38
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The development of methods for the *in vitro* study of vascular interfacial transport mechanics.

Previous Serial Numbers: NHLI-42

Principal Investigator: Donald L. Fry, M.D.

Other Investigators: Victor J. Ferrans, M.D., Ph.D., and John T. Flaherty, M.D.

Cooperating Units: Section on Pathology, Cardiology Branch

Project Description:

Objective: To develop methods for the *in vitro* study of intimal transport mechanics.

Methods Employed: Arterial segments are removed and placed in a special tissue holding device (described in previous reports). The essential purpose of the device is to permit selected areas of the opened vascular interface to be studied in a controlled, thermal, chemical and mechanical milieu. The surface is exposed to serum containing various tagged molecules of interest for selected periods of time, following which the amount of tagged material that has entered the interface is estimated using reflectometric, fluorescent, or radioactivity measurements.

Major Findings: The major barrier to protein transport appears to reside in a thin layer associated with the endothelial-cell surface. This barrier appears to remain intact for periods up to four hours, so long as the endothelial surface remains covered with autologous serum. Barrier function is decreased by exposure of the endothelial surface to saline, albumin solutions, solutions containing traces of various polar solvents, such as ethanol, and solutions containing various anesthetic agents, such as nembutal.

Significance to Bio-medical Research and the Program of the Institute: These studies have shown that it is feasible to study protein transport across the vascular interface in a controlled *in vitro* situation for periods up to 4 hours.

Proposed Course of the Project: This methodology is to be applied to a wide variety of questions concerning those factors which influence the permeability of the arterial vascular interface to various molecular species of interest and in particular to proteins and lipids.

Honors and Awards: None.

Publications: None.

Serial No. NHLI-39
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of canine and miniature swine experimental atherosclerotic animal colonies

Previous Serial Number: NHLI-45

Principal Investigator: John T. Flaherty, M.D.

Other Investigators: Joseph E. Pierce, DVM, Donald L. Fry, M.D.

Cooperating Units: Laboratory of Kidney and Electrolyte Metabolism, NHLI, and Food and Drug Administration

Project Description:

Objective: To develop colonies of dogs and swine with experimentally induced atherosclerosis to be used as experimental models of human atherosclerosis. To produce atheroma by various surgical interventions. To evaluate the effect of hypothyroidism on the pattern of atherosclerosis in minipigs.

Methods Employed: An atherogenic diet was used consisting of cholesterol, cholic acid, 6-propylthiouracil and cottonseed oil. A group of 32 dogs was thyroidectomized and then placed on the diet. Serum cholesterol, triglycerides, lipoprotein electrophoretic patterns and thyroid function were monitored at monthly intervals. Arteriovenous shunts were placed in the femoral artery in 7 dogs and in the carotid artery in 8 dogs. Thoracic aorta coarctation was performed on 8 dogs. Minipigs were a) thyroidectomized and placed on an atherogenic diet (3), b) placed on the atherogenic diet only (2) and c) placed on a standard swine diet for control purposes.

Major Findings: The thyroidectomized dogs showed a marked rise in serum cholesterol, triglycerides and β-lipoproteins. Swine also developed elevations of β-lipoproteins on the atherogenic diet both with and without thyroidectomy. Arteriovenous shunts remained patent for 3-4 months.

Significance to Bio-medical Research and the Program of the Institute: The establishment of canine and miniature swine colonies with experimentally produced atherosclerosis is essential to studies of the pathogenesis of arteriosclerotic cardiovascular disease. The evaluation of the role of hypothyroidism in the localization of atheroma in minipigs is important in the interpretation of the data that can be obtained from hypothyroid atherosclerotic dogs, as well as for evaluation of the role of other tissue factors involved in the localization of lipid deposition.

Proposed Course of the Project: Further comparative studies of the detailed topography of lipid deposition, collagen deposition and smooth muscle

proliferation will be carried out to elucidate the role of tissue factors. The interrelations of these processes as well as their relation to mechanical factors will be studied. Based on our initial observations of femoral artery shunt compared to carotid artery shunts, there is a relative resistance seen in the common carotid artery to the build-up of lipid deposits under similar hemodynamic conditions which produce massive atheroma in the femoral artery.

Honors and Awards: None

Publications:

Flaherty, J.T., Ferrans, V.J., Pierce, J.E., Carew, T.E., and Fry, D.L.: Localizing factors in experimental atherosclerosis. Chapter in Coronary Heart Disease and Atherosclerosis. Ed. W. Likoff. Grune and Stratton, New York, 1971.

Serial No. NHLI-40
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The topography of experimental atherosclerotic lesions in the dog.

Previous Serial Number: NHLI-43

Principal Investigator: John T. Flaherty, M.D.

Other Investigators: Donald L. Fry, M.D., Joseph E. Pierce, DVM, and Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: Laboratory of Kidney and Electrolyte Metabolism, NHLI, Section on Pathology, Cardiology Branch, NHLI

Project Description:

Objective: 1. To study the topography of atheroma in hyperlipemic dogs.
2. To produce atheroma by altering flow conditions surgically.

Methods Employed: The atherosclerotic dogs were given Evans blue dye as a visual tag for albumin one hour prior to sacrificing. The entire arterial tree was removed and mounted for examination of the intimal surface following Sudan IV staining. Photographic records were made of the Sudan-stained lipid accumulations and the blue stained-albumin concentrations. Histologic sections were obtained of the principal lesions. Arteriovenous shunts or coarctations were surgically induced prior to the starting of the atherogenic diet in a second group of dogs. The albumin and lipid staining patterns were recorded and flow and pressure distribution measurements obtained prior to sacrifice.

Major Findings: Discrete lipid infiltrations were found principally at branch points and entrance regions. In early disease Evans blue patterns were similar. Histologic examination of the early lesions revealed localization of lipid in the intima often localized to endothelial cells or to the region of the internal elastic lamina. Special connective tissue stains revealed the presence of focal fibromuscular proliferations at flow dividers. The presence of collagen in apical regions and within intimal pads appeared to alter the patterns of lipid infiltration and deposition in the vessel wall. Arteriovenous shunts in the femoral artery produced extensive but characteristic atheroma, compared to comparable shunts in the carotid artery, which produced only minimal atheromas. Carotid shunts did produce fibromuscular intimal thickening in the region of the shunted artery upstream from the shunt. Coarctations produced atheroma on the leading slope of the narrowed segment.

Significance to Bio-medical Research and the Program of the Institute: The relation of the topography of albumin and lipid accumulations as well as

connective tissue changes to areas in the vascular system where mechanical stresses would be high suggests a causal relationship.

Proposed Course of the Project: Detailed topography of the atheroma will point to areas in the vascular tree where shear stresses and flow patterns should be further studied in vivo and in models. Experimentally induced atheroma will show the effect of controlled alteration of hemodynamics on lipid accumulation and fibromuscular proliferation in vessel walls.

Honors and Awards: None

Publications:

Flaherty, J.T., Ferrans, V.J., Pierce, J.E., Carew, T.E., and Fry, D.L.: Localizing factors in experimental atherosclerosis. Chapter in Coronary Heart Disease and Atherosclerosis. Ed. W. Likoff. Grune and Stratton, New York, 1971.

Serial No. NHLI-41
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Endothelial nuclear orientation and morphology and its relation to hemodynamic factors

Previous Serial Number: NHLI-44

Principal Investigator: John T. Flaherty, M.D.

Other Investigators: Dali J. Patel, M.D., Ph.D., Joseph E. Pierce, DVM, Victor J. Ferrans, M.D., Ph.D.

Cooperating Units: Laboratory of Kidney and Electrolyte Metabolism, NHLI, and Section on Pathology, Cardiology Branch, NHLI

Project Description:

Objective: To study the variations in nuclear orientation, and morphology of canine arterial endothelium and to relate these to shear stresses and secondary flow patterns. To evaluate the role of physiologic shear stresses in orienting and molding the nuclei. Surgical transposition of segments of arteries to study the effect of physiologic shear stresses in reorienting and reshaping endothelial nuclei in a chronic in vivo preparation.

Methods Employed: Arterial trees from normal dogs were removed, opened longitudinally and stretched to in vivo dimensions. The surface was then washed with isopropyl alcohol and the nuclei stained with Evans blue dye. Photomicrography was employed to make a permanent record of the endothelial cells at many selected locations along the arterial tree. Major axis orientation angles were measured with a protractor and minor axes lengths and cell-population density were recorded. Surgical transposition of segment of thoracic aorta was carried out and the endothelial patterns studied at 3, 10, 21 and 70 days.

Major Findings: Study of the variations in orientation, shape, and density of the endothelial nuclei at various locations along the arterial tree suggest a correlation between hemodynamic factors, and "normal" endothelial cell patterns. Markedly non-axial nuclear orientations are found in the aortic arch and in the upper abdominal aorta which could logically correlate with secondary flow patterns in these regions. Marked, but systematic variations of nuclear shape and density are also found, which are suggestive of a relationship to local hemodynamic factors. The orientation of endothelial nuclei on the surgically transposed segment showed complete alignment to the new flow direction in 10 days.

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Serial No. NHLI-41
1. Cardiology Branch
2. Section on Clinical Biophysics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Significance to Bio-medical Research and the Program of the Institute: The establishment of a relationship between endothelial morphology and orientation to the blood flow patterns to which it is subjected would provide a valuable tool in the study of vascular hemodynamics in animals as well as in human beings by in vitro observation.

Proposed Course of the Project: Hot-film anemometry will be employed to study the associated blood velocity profiles and shear distributions. Endothelial nuclear strain measured by nuclear deformation will be quantitated and related to hemodynamic factors in an in vitro preparation.

Honors and Awards: None

Publications: None

Serial No. NHLI-42(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Healed Valvular Infective Endocarditis

Principal Investigator: Neil A. Buchbinder, M.D.

Other Investigators: William C. Roberts, M.D.

Cooperating Units: None

Project Description: Clinical and anatomic features are described in 29 necropsy patients with healed valvular infective endocarditis. Active infective endocarditis (AIE) occurred on anatomically abnormal valves in 24 patients, 9 of whom had congenitally bicuspid aortic valves. Six patients had congestive heart failure before AIE. All 29 patients had evidence of valvular dysfunction during and after AIE, 27 of whom had overt signs of heart failure AIE was the sole or contributary cause of valvular dysfunction in at least 23 patients. Aortic valve perforation was discernable in 10 patients. 5 patients had ruptured mitral chordae tendineae. The average time from AIE to necropsy was 4.3 years. The average longevity of patients with aortic valve perforations was 7.8 years and 4.1 years for those with ruptured chordae tendineae. The absence of a "myocardial factor" specific to IE is further substantiated by the fact that only 4 of 17 patients with adequate surgical correction of valvular dysfunction died as a result of persistent congestive heart failure. Death in the other 13 postoperative patients was caused by a surgical complication and not myocardial failure. The presence of fibrosis of the papillary muscles in 83% of patients supports the view that this lesion is the result of healed papillary muscle necrosis which has been found in 75% of necropsy patients with AIE.

Honors and Awards: None

Publications: None

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Serial No. NHLI-43(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Active Non-infective Thrombotic Endocarditis

Principal Investigator: Neil A. Buchbinder, M.D.

Other Investigators: William C. Roberts

Cooperating Units: None

Project Description: The clinical and necropsy features of 45 patients with active non-infective thrombotic endocarditis (ANITE) were studied. Vegetations occurred on anatomically normal valves in 40 patients and functionally normal valves in 44 patients. Malignancy was present in 39 patients, 31 of whom were being treated with chemotherapy. A precordial murmur was present in 55% of patients. Twenty-eight patients were febrile, 9 of whom had positive blood cultures. The diagnosis was not made premortem in any patients. An embolic infarct was suspected clinically in only 3 patients but was found at necropsy in 31 patients, 14 of whom had multiple infarcts. Death in 6 patients was caused by a CNS embolus. In no patient were organisms demonstrable in histologic sections of the vegetation. Thus, bacterial implantation in vegetations of ANITE is not felt to be the underlying mechanism for the development of AIE. The possible relationship of ANITE to a coagulopathy was studied. Thrombocytopenia (75,000) was present in 25 of 31 patients. Thirteen of the 25 had a possible etiology (hematopoietic malignancy or cytotoxic drug) whereas 12 had no apparent explanation. In only 3 of these patients was a complete coagulation profile available for study. 2 of the 3 had criteria disseminated intravascular coagulation.

Honors and Awards: None

Publications: None

Serial No. NHLI-44(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Active Valvular Infective Endocarditis

Principal Investigator: Neil A Buchbinder, M.D.

Other Investigators: William C. Roberts, M.D.

Cooperating Units: None

Project Description: Clinical and anatomic features are described in 55 patients with active valvular infective endocarditis. Fifty-eight percent had vegetations on previously anatomically normal valves. Predisposing factors allowing entrance of virulent or unusual organisms, or alterations of host defense mechanisms, are believed to account for the frequency of IE on normal valves. Valvular dysfunction occurred in 70% of the 55 patients, causing congestive heart failure in all. Myocardial lesions were present in 92% of the 39 patients in whom multiple histologic sections were examined. Papillary muscle necrosis was present in 75% but contributed to mitral regurgitation in only one. Myocardial inflammation was focal in all but 2 patients. Myocardial lesions are not believed to be a primary cause of congestive heart failure. No correlation was found between congestive heart failure and any or all myocardial lesions. Pericarditis was found in 9 patients (18%), and in 8 a site of direct extension of the inflammation into the pericardium was apparent. Ring abscesses were found in 10 of 19 patients with aortic regurgitation. The most common renal lesion was infective (abscess or pyelonephritis).

Honors and Awards: None

Publications: None

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Serial No. NHLI-45(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Active Infective Endocarditis Confined to Mural Endocardium

Principal Investigator: Neil A. Buchbinder, M.D.

Other Investigators: William C. Roberts, M.D.

Cooperating Units: None

Project Description: The clinical and necropsy features of 5 patients with active infective endocarditis confined to non-valvular (mural) endocardium were studied. Each patient had an underlying malignant disease; 4 had leukemia and 1 lymphoma. The causative organism was fungal in each; Candida in 3, Phycomycetes in 1 and Aspergillus in 1. The vegetations were located on the right ventricular endocardium in 3 patients and left atrial endocardium in 2. Only one patient had a precordial murmur and this was thought to be functional. The diagnosis of fungal infection was made in each patient but the diagnosis of endocarditis was not made in any. The pathogenesis of this unusual form of infective endocarditis was studied. In each patient the endocardial lesion was not primary but rather the result of extension of an infective process originating at another site. Each of the 3 patients with vegetations on the right ventricular endocardium had numerous myocardial abscesses as part of systemic fungal septicemia. The endocardial vegetations in these patients resulted from extension of a myocardial abscess to involve the endocardium. The 2 patients with IE of left atrial mural endocardium had extensive fungal infection of the lungs with fungal thrombi in pulmonary vein vegetations into the left atrium. No definite evidence of cardiac dysfunction was found in any patient although the left atrial vegetation in one patient was extensive enough to have decreased pulmonary venous return.

Honors and Awards: None

Publications: None

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Serial No. NHLI-46(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Spleen in Type I Hyperlipoproteinemia: Histochemical, Biochemical, Microfluorimetric and Electron Microscopic Observations

Previous Serial Number: NHLI-74(c)

Principal Investigator: Victor J. Ferrans, M.D.

Other Investigators: L. Maximilian Buja, M.D.
William C. Roberts, M.D.
Donald S. Fredrickson, M.D.

Cooperating Units: Molecular Disease Branch, National Heart and Lung Institute

Project Description: Histochemical, biochemical, microfluorimetric and electron microscopic studies were made of the spleen of a patient with type I hyperlipoproteinemia. Foam cells were observed that contained a material identified as ceroid on the basis of its autofluorescence, acid-fastness, sudanophilia, PAS-positivity and insolubility in organic solvents. Electron microscopy showed that the ceroid was organized in the form of granules with concentric lamellae of irregular periodicity. The process of formation of these granules was described in detail. The ceroid was considered to represent non-digestible end-products of the metabolism of chylomicrons taken up by macrophages in splenic sinusoids.

Honors and Awards: None

Publications: Ferrans, V. J., Buja, L. M., Roberts, W. C., and Fredrickson, D. S.: The spleen in type I hyperlipoproteinemia: Histochemical, biochemical, microfluorimetric and electron microscopic observations. Am. J. Path. (In press).

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Serial No. NHLI-47(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Ultrastructural Studies of Cardiac Biopsies in Primary Myocardial Disease

Previous Serial Number: NHLI-76(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: William C. Roberts, M.D.
Rashid Massumi, M.D.*
Nayab Ali, M.D.*
Gerald Shugoll, M.D.**

Cooperating Units: *Cardiology Division, D. C. General Hospital
**Cardiology Service, Veterans Administration Hospital, Washington, D.C.

Project Description: Electron microscopic studies are being made of cardiac biopsies obtained from patients with primary myocardial disease at D.C. General Hospital and V. A. Hospital, Washington, D.C. A Konno catheter is being used to biopsy the septal wall of the right ventricle. Observations made indicate that mitochondrial alterations, swelling of the sarcoplasmic reticulum and dilatation of the transverse tubular system are the main alterations present.

Honors and Awards: None

Publications: Data to be presented at Sept., 1971, International Symposium on Cardiomyopathies, will be published in a book which will contain the proceedings of this meeting.

Serial No. NHLI-48(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Electron Microscopic and Histochemical Studies of the Liver in GM_1 Gangliosidosis

Previous Serial Number: NHLI-73(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: Donald S. Fredrickson, M.D.
Howard Sloan, M.D.

Cooperating Units: Laboratory of Molecular Diseases, National Heart & Lung Institute

Project Description: Ultrastructural and histochemical studies have been made on liver tissue obtained from 2 patients with biochemically proven GM_1 gangliosidosis. Results obtained indicate that two different compounds are stored in the liver: GM_1 ganglioside, which is localized in the hepatocytes, where it forms membranous cytoplasmic bodies that are probably of lysosomal origin, and an acid mucopolysaccharide-like material, which is present within the lysosomes of the Kupfer cells in the form of thin-walled, straight tubules that measure from 170 to 200 Å in diameter. The material from which the tubules are derived accumulates at first in the endoplasmic reticulum of the Kupfer cells and is subsequently transferred to the lysosomes, where the tubules develop.

The storage of two different compounds in two different cell types of the same organ is of great interest in view of the currently held concept of a single enzyme deficiency in this disorder. It is possible that both compounds share a common path of degradation.

Honors and Awards: None

Publications: To be prepared for publication.

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Serial No. NHLI-49(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Histochemical and Electron Microscopic Study of Cardiac Myxomas

Previous Serial Number: NHLI-75(c)

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: William C. Roberts, M.D.

Cooperating Units: None

Project Description: Histochemical studies are being made on 8 cardiac myxomas, and electron microscopic observations have been made on 2. The electron microscopic data support the concept that the tumor cells are derived from endocardial endothelial cells. The myxoma cells show a marked tendency to form capillary- or duct-like structures. Their cytoplasm contains numerous filaments that measure 50-70 Å in diameter and are similar to those described in normal endothelial cells. The myxoma cells also contain variable amounts of rough-surfaced endoplasmic reticulum and iron particles. The stroma of the tumor is composed of a material the structure of which varies from amorphous to finely fibrillar. The histochemical studies are primarily concerned with the histochemical characterization of the carbohydrate components of the myxomatous stroma.

Honors and Awards: None

Publications: None

Serial No. NHLI-50
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Hyperosmotic Perfusate on Ultrastructure and Function of the Isolated Canine Heart

Previous Serial Number: None

Principal Investigator: Victor J. Ferrans, M.D., Ph.D.

Other Investigators: L. Maximilian Buja, M.D.
Sidney Levitsky, M.D.
William C. Roberts, M.D.

Cooperating Units: Surgery Branch, National Heart and Lung Institute

Project Description: Ultrastructural and functional observations are described on isolated canine hearts preserved for 18 hours with either filtered plasma perfusate (5 dogs) or with filtered plasma-dextran perfusate (6 dogs). Interstitial edema, swelling of sarcoplasmic reticulum, and mitochondrial damage were observed in each of the 5 hearts perfused by filtered plasma. In contrast, interstitial edema was absent in each of the 6 hearts perfused by filtered plasma-dextran, and swelling of sarcoplasmic reticulum and mitochondrial damage occurred in only 2. Myocardial compliance also appeared to be better in hearts perfused by filtered plasma-dextran than in hearts perfused with filtered plasma. In conclusion, the osmolarity of the perfusate is important in preventing edema in preserved hearts.

Honors and Awards: None

Publications: Ferrans, V.J., Buja, L.M., Levitsky, S., and Roberts, W.C.: Effects of hyperosmotic perfusate on ultrastructure and function of the isolated canine heart. Laboratory Investigation. In press.

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Serial No. NHLI-51(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Papillary Muscle Dysfunction Before and After Auscultatory Mitral Regurgitation. Hemodynamic and Morphologic Documentation

Principal Investigator: William C. Roberts, M.D.

Other Investigators: M. Wayne Falcone, M.D.
James A. Ronan, Jr., M.D.

Cooperating Units: Department of Medicine, Georgetown University School of Medicine, Division of Cardiology, Georgetown University Hospital, Washington, D.C.

Project Description: Clinical, hemodynamic and anatomic findings are described in a 71-year-old man with "silent" acute myocardial infarction. Attention is called to the hemodynamic documentation of mitral regurgitation resulting from papillary muscle necrosis both shortly before and shortly after clinical appearance of a murmur of mitral regurgitation. The interest of this case is the fact that the patient underwent 2 cardiac catheterizations during the time that the myocardium was being infarcted. It was thus possible to demonstrate the hemodynamic consequences of papillary muscle necrosis. It was shown that hemodynamic evidence of mitral regurgitation was present before auscultatory evidence of mitral regurgitation appeared.

Honors and Awards: None

Publications: None

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Serial No. NHLI-52(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Aneurysm of the Non-patent Ductus Arteriosus.

Previous Serial No. None

Principal Investigator: William C. Roberts, M.D.

Other Investigators: M. Wayne Falcone, M.D.
Joseph K. Perloff, M.D.

Cooperating Units: Department of Medicine, Georgetown University School of Medicine, Division of Cardiology, Georgetown University Hospital, Washington, D.C.

Project Description: Clinical and necropsy findings are described in a 6-week-old infant with aneurysm of the ductus arteriosus. It was obliterated at the pulmonary arterial end and patent at the aortic end. Observations in 60 previously described patients with ductal aneurysm disclosed that 46 were in infants less than 2 months old, 4 in children, and 11 in adults. The aortic end of the ductal aneurysm is always patent. The pulmonary arterial end may or may not be patent. Since nearly half of the ductal aneurysms tend to develop complications (rupture, embolism or infection) operative resection appears indicated.

Honors and Awards: None

Publications: Falcone, M. W., Perloff, J. K., and Roberts, W. C.: Aneurysm of the non-patent ductus arteriosus. Am. J. Cardiol. In press.

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Serial No. NHLI-53(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Congenital Aortic Stenosis Resulting from a Unicommissural Valve. Clinical and Anatomic Features in 21 Adult Patients.

Principal Investigator: William C. Roberts, M.D.

Other Investigators: M. Wayne Falcone, M.D.
Andrew G. Morrow, M.D.
Joseph K. Perloff, M.D.

Cooperating Units: Surgery Branch, National Heart and Lung Institute and Department of Medicine, Georgetown University School of Medicine, Division of Cardiology, Georgetown University Hospital, Washington, D.C.

Project Description: Clinical, electrocardiographic, phonocardiographic, radiographic, hemodynamic and anatomic findings are presented in 21 adult patients with stenotic unicommissural aortic valves. Distinction of congenitally unicuspid and bicuspid aortic valves before operation or autopsy was not possible. Although the basic structure of the valve may render it inherently stenotic, the age at which a murmur was first noted (avg. 19 years), the duration of a known murmur (avg. 25 years), and the age of onset of first symptoms of left ventricular outflow obstruction (avg. 41 years) strongly suggest that stenosis at least in part is acquired. The relationship of the true and false commissures to the coronary arterial ostia could be determined with certainty in 12 patients. The basic division of the aortic valve into left, right, and non-coronary cusps is maintained, but the raphes do not extend to the valve orifice. Because the aortic valve is attached to the ascending aorta at only one point (the true commissure), which is at the level of the orifice, valvotomy is hazardous, and valve replacement appears indicated when operative treatment becomes necessary in the adult patient with a stenotic unicommissural aortic valve.

Honors and Awards: None

Publications: Falcone, M. W., Roberts, W. C., Morrow, A. G., and Perloff, J. G.: Congenital aortic stenosis resulting from a unicommissural valve: Clinical and anatomic features in 21 adult patients. Circulation. In press.

Serial No. NHLI-54
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Acute and Chronic Effects of Normothermic Anoxia on Canine Hearts: Light and Electron Microscopic Evaluation

Previous Serial Number:

Principal Investigator: L. Maximilian Buja, M.D.

Other Investigators: Sidney Levitsky, M.D.
Victor J. Ferrans, M.D., Ph.D.
Sherman G. Souther, M.D.
William C. Roberts, M.D.
Andrew G. Morrow, M.D.

Cooperating Units: Surgery Branch, National Heart and Lung Institute

Project Description: To evaluate the acute and chronic effects of normothermic cardiac anoxia, structural and functional observations were made on the hearts of 29 dogs subjected to total cardiopulmonary bypass. Elective, normothermic cardiac arrest was induced by aortic cross-clamping for 30 minutes in 7 dogs, and for 45 minutes in 16 dogs. The remaining 6 dogs, which served as controls, were placed on bypass without aortic cross-clamping. The 6 control dogs and 6 of 7 dogs subjected to 30 minutes of cardiac anoxic showed excellent cardiac function and minimal myocardial damage; one dog in the latter group that died 6-12 hours after bypass showed no myocardial damage. All 16 dogs subjected to 45 minutes of cardiac anoxia showed extensive myocardial damage. Depressed cardiac function was demonstrated in 3 of 5 dogs that survived for 8 days or longer after bypass. The acute cardiac damage was of the myofibrillar degeneration type and progressed to replacement fibrosis; this damage was selectively localized in the left ventricular papillary muscles and subendocardium.

Honors and Awards: None

Publications: Buja, L. M., Levitsky, S., Ferrans, V. J., Souther, S. G., Roberts, W. C., and Morrow, A. G.: Acute and chronic effects of normothermic anoxia on canine hearts: Light and electron microscopic evaluation. Suppl. to Circulation 43 and 44: In press.

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Serial No. NHLI-55(c)
1. Cardiology Branch
2. Section of Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Causes of Death and Other Anatomic Observations after Cardiac Valve Replacement

Principal Investigator: William C. Roberts, M.D.

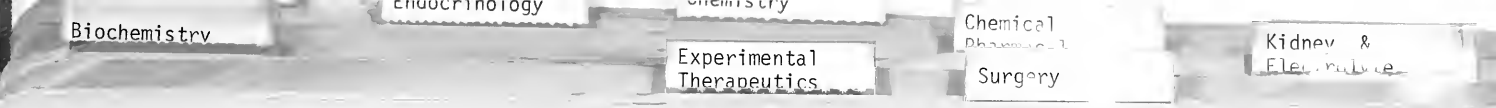
Other Investigators: Andrew G. Morrow, M.D.

Cooperating Units: Surgery Branch, National Heart and Lung Institute

Project Description: From 1963 through 1970, 181 patients died following replacement of 1 or more cardiac valves with prostheses. Each patient was studied at necropsy. The patients were divided into two groups: 1) 107 patients who died within 2 months of operation (early deaths), and 2) 74 patients who died at later periods up to 80 months (late deaths). Of the 220 cardiac valves replaced in these 181 patients, Starr-Edwards prostheses were used in 200. Among the causes of death early prosthetic dysfunction was responsible in 30%, no anatomic cause was found in 28%, bleeding and technical mishaps 19%, central nervous system catastrophies 7%, associated undiagnosed or uncorrected valve disease 7%, infection 4%, and miscellaneous causes 5%. Among the 74 patients who died late, prosthetic dysfunction accounted for death in 37% and factors secondarily related to the prosthesis in 24%. These latter factors included systemic embolism, CNS hemorrhage from hypoprothrombinemia, secondary endocardial fibrosis of the left ventricle, secondary intimal fibrosis of the ascending aorta, and hepatitis. In 18% of the late deaths the cause was undetermined, in 16% death was related to the underlying cardiac disease and in 5% the cause of death was unrelated to the heart. Although additional years of life have been provided to many critically ill patients from severe valvular heart disease by cardiac prostheses it is apparent 10 years after valve replacement that the ideal cardiac valve is not presently available. It would appear from this study and others that the rigid frame prostheses are simply not capable of functioning properly in a few selected hearts with either small left ventricular cavities or small aortas. Whether or not intimal proliferation which occurs in the aortic root late following aortic valve replacement and also in the area of the coronary arterial ostia will lead to chronic myocardial ischemia remains to be seen. The degree of intimal proliferation in the ascending aorta in the late deaths corresponded to the presence or absence and degree of renal hemosiderosis indicating that the aortic lesion is due to turbulence of blood transversing the prosthetic valve and that this turbulence causes intravascular hemolysis which is apparent in the kidney by deposition of iron in cytoplasm of renal tubules. Whether or not the metallic hollow ball used in Starr-Edwards prostheses will hold up over many years still remains to be seen, but there is evidence that the cloth-covered struts do show evidence of wearing after about three years.

Honors and Awards: None

Publications: Roberts, W. C. and Morrow, A. G.; in Vogel, J. (Ed.), Long-term prognosis following valve replacement. Second Conference on Cardiovascular Disease. Basel, Switzerland, S. Karger. Publication date 1971.


Biochemistry
Endocrinology
Experimental Therapeutics
Surgery

ANNUAL REPORT OF THE
CLINIC OF SURGERY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

The clinical and laboratory programs of the Surgery Branch have, as in past years, largely centered upon the study of operative methods for the correction of congenital and acquired heart and lung diseases, assessment of the results of such operations, and laboratory studies related to cardiovascular physiology and pharmacology.

Radioisotope-powered Pacemakers: A previous report has described the initial research effort to develop a cardiac pacemaker powered by an isotopic heat source. This work has been carried out with the cooperation and support of the Atomic Energy Commission. The pacemakers, fueled with metallic ^{238}plutonium, were designed to have a useful life of more than ten years, to obviate the frequent power pack changes which are necessary with pacemakers powered by conventional batteries. After initial laboratory and bench testing eight fueled pacemakers were inserted in normal dogs. Serial observations in these animals indicated that after an initial stabilization period the pacemaker rate and the amplitude and configuration of the pacing artifact remained unchanged. Over a period of 18 months, however, pacing failed in every animal after an average time of eight months. In each case the electrode was shown to be physically and functionally intact after the pacemaker was disconnected from it. Examination of the pacemakers which were removed has revealed the cause of failure in seven of the eight units to be a defective output transistor in the pacemaker circuit. In the single unit in which the nuclear power supply was defective, failure resulted from detachment of one of the thermocouple tapes. Needless to say, this experience was discouraging, but reinforced the research plan which emphasizes extensive animal evaluation before the units are utilized in man. A new series of pacemakers incorporating an improved pacemaker circuit will be made available for animal implantation early this year. The experience with the modified pacemakers will determine the likelihood of suitability for use in patients with complete heart block.

Prosthetic Heart Valves: A continuing interest of the Surgery Branch has been the evaluation, operative treatment, and subsequent followup of patients in whom replacement of one or more cardiac valves is necessary. The original Starr-Edwards prostheses, both mitral and aortic, were constructed in such a manner that much bare metal was exposed to blood, and there was a large area of interface between metal and the fabric sewing ring. During 1964-66 experimental studies revealed that if all the metallic surfaces were covered with a thin layer of fabric, the fabric would become convered with host neointima, and no foreign material except the ball was exposed to blood. This principal was incorporated into valves produced commercially during 1966-68, and aortic prostheses (series 2300) and mitral prostheses (series 6300) were implanted in approximately 100 patients. Subsequent evaluation of these patients by both clinical examination and serial cardiac catheterizations revealed that the valves had poor hemodynamic characteristics. Peak systolic pressure gradients in excess of 50 mm. Hg were common after aortic valve replacement, and gradients as high as 75 mm. Hg were sometimes recorded. Similarly, in the mitral position the 6300 valves almost never permitted the left atrial pres-

sure to fall to a normal level, and significant diastolic gradients were invariably seen. Also, in the aortic position these valves caused severe hemolytic anemia in a number of patients, even when perivalvular regurgitation was not present. This experience led to the development of an improved series of cloth-covered prostheses in which the ratio of orifice area to ball diameter was increased by nearly 20 percent. Over the past two years these valves (aortic - 2310, mitral -6310) have been evaluated in another 100 patients. Postoperative hemodynamic assessments have indicated significant improvement in comparison to the 2300 - 6300 series. In the aortic position peak pressure gradients of 0 to 20 mm. Hg are usual, and in the mitral position the hemodynamic picture is essentially identical to that observed with the original model 6120 (silastic ball) valves.

It has been hoped that the fabric covering utilized in both the 2300-6300 and 2310-6310 valves would result in a significant reduction in thromboembolism. To date this hope has been borne out when these valves have been utilized in the aortic position. Anticoagulants have been administered for the first six months after operation and discontinued thereafter. Only two patients are known to have sustained systemic emboli after cessation of anticoagulation. Such encouraging results have not been observed with fabric-covered valves utilized in the mitral position. Thus, systemic emboli occurred in each of the first three patients with 6310 mitral prostheses within days after anticoagulants were stopped, and in one patient the embolus proved fatal. At the present anticoagulation is stopped six months after aortic valve replacement, but continued indefinitely in all patients with mitral prostheses.

Tissue Heart Valves: In an attempt to obviate some of the disadvantages of ball or disc mechanical prostheses an initial laboratory and clinical effort to construct satisfactory heart valves of homologous or heterologous tissue have been made. At other institutions porcine aortic valves have been used for valve replacement in man after they were sterilized and fixed in formalin and mounted on a rigid metallic or plastic frame. The hemodynamic function of such valves has been generally good, and they have not been the source of emboli even though anticoagulants were not administered. Frequently, however, such valves have failed when the tissue tore away from its attachment to the rigid frame. A new valve frame has been designed in a cooperative project with the Hancock Laboratories. This frame is presently constructed of polypropylene, and the legs to which the commissures of the tissue valve are attached are made semi-flexible. Tissue valves mounted on rigid and semi-flexible frames were studied in a pulse duplicator while the stress on the sutures anchoring the valve to the frame was constantly recorded with a force transducer. With a rigid frame the stress on the attachment rose strikingly as the valve was subjected to increased closing pressure. With the semi-rigid frame, however, the slight centripital movement of the struts prevented this tension increase and, in fact, stress at maximum closing pressure was sometimes observed to be less than that at a lower pressure.

Heterologous (porcine) valves, preserved and sterilized with glutaraldehyde, and constructed on such semi-flexible frames have been utilized for mitral and/or tricuspid valve replacement in 20 patients. It has become obvious that a distinct advantage of such valves is that they can be inserted

into a diminutive left ventricular chamber such as is encountered in patients with severe calcific mitral stenosis or those with mitral disease and associated aortic stenosis. Postoperative hemodynamic studies have been carried out at 4-6 months and the function of the tissue valves is comparable to those of the 6310 ball valves. There have been no instances of thromboembolism or infection. Anticoagulants have not been administered. Only continuing clinical followup will indicate whether tissue valves of this type are sufficiently durable to recommend their general use.

Research in Organ Transplantation: A program of research in transplantation biology, with special emphasis on cardiac transplantation, has been instituted. Initial efforts have been directed toward definition of selected fundamental problems utilizing a heterotopic (abdominal) heart transplant model in inbred rats.

Immunologic enhancement (i.e. prolongation of graft survival by antibody) of Brown Norway (BN) hearts transplanted into Lewis (L) hosts has been demonstrated. Control BN hearts in L hosts were rejected in 6.4 ± 0.7 (S.D.) days. Pretreatment of L recipients with 10^7 BN spleen cells given intravenously one week pretransplant caused prolongation of graft survival to 11.7 ± 3.3 days ($P < .01$). Serum obtained from L rats injected with 10^7 BN spleen cells, when injected into normal L rats at the time of transplantation and for a short time thereafter, caused prolongation of graft survival to 11.0 ± 2.4 days. Thus, immunologic enhancement of cardiac grafts has been demonstrated, although the duration of graft prolongation is less than that previously shown for renal grafts in a qualitatively similar system.

Some in-vitro properties of the serum factor (S) responsible for graft enhancement in this system have been examined. No cytotoxicity can be detected in microcytotoxicity tests. However, serum from BN spleen cell-treated L rats does cause significant, reproducible, and immunologically specific depression of unidirectional mixed leukocyte reactions (MLR) between L and irradiated BN spleen cells. Similar results were observed even when presensitized L spleen cells were used. The depression of MLR was dose-dependent. These observations support the concept of "peripheral masking" of antigen, rather than central tolerance, as the mechanism of enhancement. Furthermore, the results suggest that this assay (i.e. effect of serum from immunized subjects on MLR) may be used as a semi-quantitative assay for the presence of enhancing antibody.

In another series of L rats receiving BN heart grafts, recipients were sacrificed at daily intervals on postoperative days one through six. Spleen cells from the transplanted L hosts were examined for DNA synthesis, utilizing a two hour incubation, and measuring the uptake of tritiated thymidine. Significantly increased ^3H- thymidine uptake, as compared to controls and sham-operated animals, was noted on postoperative day three, increasing progressively through day six. Changes in graft status, as determined by palpation and EKG activity, were inconsistent and were invariably preceded by increased in-vitro leukocyte DNA synthesis by at least 48 hours. This study suggests a rapid convenient, and probably specific assay for graft rejections which is qualitatively different from the usual clinical tests.

Also under examination are the anatomic and physiologic consequences of orthotopic cardiac graft rejection. At the time of transplantation a left ventricular pressure transducer, ascending aortic flow probe, and small piezoelectric crystals on the left ventricular endocardial surface, across the maximum transverse diameter, are implanted. Postoperatively, EKG, heart rate, LV pressure, and dp/dt, cardiac output, stroke volume, LV diameter, circumferential fiber velocity, and diastolic pressure-volume relationships are examined serially at rest and during treadmill exercise. In this way, the pathophysiology of graft rejection and the effects of immunosuppressive treatment may be defined. These studies are in progress.

Serial No. NHLI-56(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of a percutaneous transthoracic pacing wire for both routine and emergency use.

Previous Serial No.: None

Principal Investigators: Thomas Q. Winter, M. D.
Robert L. Reis, M. D.

Project Description: The two customary methods of delivering electrical impulses from a battery pack to the heart are (1) transvenous electrodes and (2) epicardial electrodes. The former has the advantage of being inserted under local anesthesia, but suffers from a failure rate of from 10% to 25% due to the tip of the electrode being ejected or dislodged from the apex of the right ventricle. This necessitates re-manipulation of the electrode to restore pacing. The epicardial electrode system has proved much more reliable, but requires a thoracotomy under general anesthesia for installation. We have tried to design a system that would combine the reliability of the epicardial system with the ease of insertion of the transvenous system. This system employs a 17 gauge needle to make a left or right ventricular puncture. A wire with a preformed tip is then introduced through the needle into the ventricular chamber. The needle is withdrawn, and the wire is gently snugged into the myocardium. For emergency use the wire would be directly connected to the pulse generator; for permanent use the wire would be tunneled subcutaneously to the pulse generator, which would also lie subcutaneously. One theoretical advantage of this system is that pacing thresholds from wires actually lodged in the myocardium are less than endocardial thresholds, thus allowing a lower setting on the pulse generator and longer battery life. Working with the Elgiloy Company of Elgin, Ill. several different wire sizes were evaluated (.006, .007, .008, .0085, .009, and .010). The latter proved to be the most desirable. Wires were coated with a polyurethane elastomer (Lycra) by the Plastics Unit of the Instrument Fabrication Section, and were introduced into dog hearts in a variety of ways. It was possible to pace the dog hearts at a low threshold (< 3.5 ma). Wires have been left in the dogs for a period of up to 4 months. Recent x-rays reveal no wire fracture. This system was also used to permanently pace one patient, a 17 year old girl with complete heart block following repair of a partial A-V canal. After nine weeks of satisfactory functioning, the wire fractured at a spot where it crossed a rib (and thus was subject to repeated flexion through a narrow radius). The wire was removed after it fractured and a transvenous electrode was inserted. The wire has been used in one emergency situation where it was necessary to pace the heart immediately. The wire was inserted quickly and triggered ventricular contractions.

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Current Status: We have a wire that is superior to any wire commercially available for emergency pacing of the heart. Only economic considerations may possibly prevent its manufacture and widespread use. Its use as a substitute for either the transvenous or epicardial electrode systems will depend on how reliable the electrode will prove to be.

Proposed course: An attempt should be made to find a manufacturer for the wire.

Serial No. NHLI-57(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Preoperative assessment of aortic and mitral valve sizes to facilitate valve replacement with autogenous tissue valves.

Previous Serial Number: None

Principal Investigators: Thomas Q. Winter, M. D.
D. Luke Glancy, M. D.
Robert L. Reis, M. D.
Andrew G. Morrow, M. D.

Cooperating Unit: Cardiology Branch, NHLI

Project Description: To reduce the time of aortic or mitral valve replacement when autogenous tissue valves are utilized it is essential that a preoperative assessement of approximately what size valve will be required, so that one surgeon can construct the valve while another opens the chest and prepares the operative site. To gain experience in predicting the valve size, all preoperative left-ventricular and aortic root cines which had accompanying measuring grids were reviewed.

Measurements were made at the area of the annulus, at the sinus of Valsalva area, and at the narrowest part of the ascending aorta. Measurements were corrected for magnification error, and the results were correlated with the actual valve size subsequently employed at operation. The measurement made at the annulus was the most valuable in predicting the size of the valve actually used. Using this measurement, the correct valve size was predictable in the majority of cases analyzed, and the predicted valve size never differed from that of the valve utilized by more than one size, well within the margin of error allowed.

To date, we have only analyzed two cines in patients who have had mitral valve replacement. It is too early to judge the usefulness of that procedure.

Proposed Course: Continue to accumulate experience and publish the results if the data for estimating the size of the mitral valve proves accurate, as this has not yet been reported.

Serial No. NHLI-58(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A new cause for diastolic murmur following caged-ball replacement of the aortic valve

Previous Serial Number: None

Principal Investigators: Thomas Q. Winter, M. D.
Robert L. Reis, M. D.
William C. Roberts, M. D.

Cooperating Unit: Section of Pathology, Cardiology Branch, NHLI

Project Description: A diastolic decrescendo murmur along the left sternal border following aortic valve replacement usually signifies a peri-basilar leak around the prosthetic valve. Recently a patient with a cloth-covered aortic valve was seen who manifested a diastolic murmur but at autopsy no peri-basilar leak was found. Fibrous tissue had grown into the valve seating ring making the valve relatively stenotic and at the same time providing an uneven seating surface for the metal ball, allowing a modest amount of aortic regurgitation. This has not been previously reported.

Proposed Course: Project completed. Manuscript in process of being submitted for publication.

Serial No. NHLI-59(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Four Years' experience with fabric-covered Starr-Edwards ball valves

Previous Serial Number: None

Principal Investigators: Thomas Q. Winter, M. D.
Robert L. Reis, M. D.
A. G. Morrow, M. D.

Project Description: Fabric-covered Starr-Edwards ball valves have been used for both aortic and mitral valve replacements at this Institution for over four years. Approximately 100 patients with the 2300/6300 series valves have been followed for up to four years. The followup period for the later series, the 2310/6310 series, has been approximately two years. For each group determinations of mean valve area, peak systolic gradient, and left atrial pressure are being made from the postoperative catheterization data. The incidence of emboli in terms of patient months will be given for each group. These data will be stored on a Wylbur Data Set and be available for future reference, as well as serving as the initial batch of data in the Retrospective Study of Cardiac Valve Replacement which was begun approximately one year ago. (The data from the 230 patients are presently being transferred to key punch cards; for this reason specific numbers are not available at this time.)

Proposed Course: The information gathered in the above study will be submitted for publication. The title of the project should be changed from "Retrospective" to "Continuing" and data should be accumulated on all the prosthetic valve replacements performed at this Institution and stored in a system such as the Wylbur Data System so that it will be available for easy reference in the future.

Serial No. NHLI-60
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An exploration of the usefulness of secondary parameters in improving accuracy of stroke volume estimation by computer analysis of aortic pulse contour.

Previous Serial No.: None

Principal Investigators: David B. Melvin, M. D.
Kenneth Kempner, M. A.
Robert L. Reis, M. D.

Cooperating Unit: Computer Systems Laboratory, DCRT

Project Description: Many formulas have been proposed for the estimation of cardiac stroke volume, and thus cardiac output, by computer analysis of aortic pulse contour. Several of these have been of great clinical usefulness. A critical limitation experimentally and clinically has been a decreasing degree of accuracy with abrupt changes in peripheral vascular resistance. While changes of this degree are not common, it is precisely during such periods that a rapid and reliable evaluation of cardiac output is most needed. It was felt that by monitoring other parameters simultaneously, a pattern change suggestive of, and quantitatively related to, these systematic errors in current methods might be detected.

Eight dogs were studied. Continuous records were made of 1) aortic root flow by electromagnetic flowmeter, 2) aortic root instantaneous pressure, 3) left femoral arterial pressure, 4) right femoral flow by a Doppler ultrasonic flow probe, and 5) right femoral flow by an electromagnetic flow probe. Recordings were made on electromagnetic tape. The dogs were given a series of drugs to alter cardiac and peripheral vascular function. Partial venous inflow occlusion was effected briefly during each pharmacologic intervention.

Proposed Course: Experimental data will be digitized for analysis. Initial computations will be 1) an estimated stroke volume for each beat by several currently used formulas, 2) the actual stroke volume by aortic electromagnetic flow probe, and 3) the errors incurred in estimation. The percentage of error (positive or negative) will be plotted against time for each entire experiment. Then, on the same axis can be plotted multiple derivations of the parameters measured -- (for example, the Doppler flow, the aorto-femoral pulse accentuation, the femoral pulse pressure, etc.). Any visually suggested correlation will be tested mathematically. A new formula with a factor incorporating the value which correlates with error will be written. The new formula will then be applied to the original curves, and error assessed again. Completion of data analysis and preparation of manuscript for publication.

Serial No. NHLI-61
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The direct and reflex effects of isolated changes in pH, pO_2, and pCO_2 upon systemic and pulmonary vascular resistance

Previous Serial No.: None

Principal Investigators: Jefferson F. Hollingsworth, M. D.
Robert L. Reis, M. D.

Other Investigators: Bradley M. Rodgers, M. D.
John W. Yarbrough, M. D.
Frederick H. Levine, M. D.
David B. Melvin, M. D.
David M. Conkle, M. D.

Project Description: The isolated effects of pH, pO_2, and pCO_2 upon systemic and pulmonary vascular resistance are incompletely defined because compensatory mechanisms tend to obscure individual changes. A canine preparation utilizing cardiopulmonary bypass and employing isolated perfusion and oxygenation of the systemic and of the pulmonary circulations has been developed. Twenty-two NIH foxhounds were studied. This preparation facilitates the investigation of isolated pH, pO_2, and pCO_2 changes upon systemic and pulmonary circulations. Utilizing this preparation, neurological pathways remain intact, but there is complete humoral isolation of the systemic and pulmonary circulations.

Results: Neither airway hypoxia, pulmonary arterial hypoxia, nor a combination of these factors significantly altered pulmonary vascular resistance. Systemic hypoxia, however, significantly increased pulmonary vascular resistance. Systemic hypercapnic acidosis with a normal pO_2 and a physiologic pulmonary circulation also significantly increased pulmonary vascular resistance. A combination of systemic hypercapnic acidosis, and hypoxia produced a synergistic increase in pulmonary vascular resistance. It is proposed that these changes in pulmonary vascular resistance are reflexly mediated since humoral isolation of the circulations was confirmed. Neither systemic hypercapnia with a physiologic pH, nor systemic acidosis with a physiologic pCO_2 produced a significant increase in pulmonary vascular resistance.

The direct effects of selective changes in the systemic pH, pO_2, and pCO_2 on systemic vascular resistance were also evaluated. Systemic hypoxia produced no change in systemic vascular resistance, however, one minute folbwing reoxygenation systemic vascular resistance significantly decreased. Systemic hypercapnia with a physiologic pH also increased systemic vascular resistance. Lactic acidosis with a constant pCO_2 did not alter systemic vascular resistance.

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July 1, 1970 through June 30, 1971

Metabolic alkalosis significantly decreased systemic vascular resistance.

Proposed Course: An investigation of the direct effect of airway and pulmonary vascular hypercapnia, acidosis, and hypercapnic acidosis upon pulmonary vascular resistance is proposed.

Serial No. NHLI-62
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Measurement of left ventricular diameter and contraction velocity by ultrasound

Previous Serial No.: None

Principal Investigators: Edward B. Stinson, M. D.
Glenn Rohmoeller
Andrew G. Morrow, M. D.

Cooperating Unit: Biomedical Engineering and Instrumentation Branch

Project Description: Internal left ventricular transverse diameter in dogs is measured continuously and instantaneously by ultrasound methods. Lead titanate-zirconate piezoelectric crystals, which oscillate at eight megahertz in the thickness mode when stimulated electrically, are cut into 6 mm. diameter disks and mounted in lucite housings. Two crystals are implanted on the endocardial surface of the left ventricle across the maximum internal transverse diameter and the electrical leads are exteriorized. One crystal is pulsed and the receiving crystal monitored. Sound travels through blood at a speed of 1.5 millimeters per microsecond. By processing the receiving amplifier output with a tracking gate, similar to the type of electronics system used by the military to track rockets, an analog output proportional to left ventricular diameter is displayed on a scope or chart recorder.

Results: Preliminary results indicate that with the tracking gate the system is practical. Several dogs have been successfully monitored during exercise.

Proposed Course: The piezoelectric crystals are being evaluated as to their antenna properties and sonic techniques are being applied to maximize beam width and range.

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Serial No. NHLI-63
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of chronic cardiac denervation on the cardiac response to exercise

Previous Serial No.: None

Principal Investigators: Edward B. Stinson, M. D.
Andrew G. Morrow, M. D.

Project description: An intact dog model has been developed to evaluate instantaneous cardiac responses to treadmill exercise. After preoperative treadmill training, animals are chronically instrumented by implantation of miniature, solid-state pressure transducers in the left ventricle and aorta, a flow transducer around the ascending aorta, piezoelectric crystals on the endocardial surface of the left ventricle across the maximum transverse diameter, and pacing wires. Experimental subjects are cardiac-denervated by regional neural ablation. After recovery from operation, the dogs are studied at rest and during exercise and the following parameters are measured directly or derived offline from magnetic tape recordings: heart rate, cardiac output, stroke volume , peak ejection velocity, acceleration of ejection, left ventricular peak systolic and end-diastolic pressure, left ventricular dp/dt, aortic phasic and mean pressure, stroke work and power, left ventricular diameter, volume, and rate of circumferential fiber shortening. The effects of heart rate control and pharmacologic autonomic blockade on exercise response in both normal and cardiac-denervated animals are also being evaluated.

Results: Preliminary results in two cardiac-denervated and three control dogs indicate that profound alterations from normal adaptive mechanisms are induced by cardiac denervation. In particular, they illustrate a major role for the Frank-Starling mechanism in the response of the denervated (and by inference, the transplanted) heart to physical stress. At present the collection and analysis of data have not been completed.

Proposed course. To be continued.

Serial No. NHLI-64
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Reports
July 1, 1970 through June 30, 1971

Project Title: The effects of chronic right heart failure on left ventricular function

Previous Serial No.: None

Principal Investigators: John W. Yarbrough, M. D.
Robert L. Reis, M. D.

Other Investigators: David Melvin, M. D.
Jefferson Hollingsworth, M. D.
David Conkle, M. D.

Project Description: Left ventricular function curves were inscribed by means of a right heart bypass preparation at constant heart rate and aortic pressure in 17 American foxhounds. In 7 dogs, severe right heart failure had been produced by creation of tricuspid regurgitation and pulmonary arterial constriction 2-3 months previously. The normal foxhounds served as controls. Left ventricular function curves were depressed in all 7 dogs with chronic right heart failure compared to the curves inscribed in the control dogs. Dilatation and hypertrophy of the right ventricle was evident in addition to hepatosplenomegaly, severe malnutrition, and ascites in each of the 7 dogs with right heart failure.

The mechanism by which chronic right heart failure produces left ventricular dysfunction has not been defined. Possibly, septal hypertrophy alters left ventricular compliance and reduces left ventricular function. Metabolic defects from malnutrition may play a more direct role.

Serial No. NHLI-65
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of glutaraldehyde-fixed fascia lata as a material for tissue valve leaflets

Previous Serial No.: None

Principal Investigators: John W. Yarbrough, M. D.
Robert L. Reis, M. D.

Other Investigators: Jefferson Hollingsworth, M. D.
David Melvin, M. D.
David Conkle, M. D.

Project description: Fascia lata was obtained from cadavers without the use of sterile technique. The fascia was immediately used to construct valves on the Reis-Hancock strut and then placed in 0.25% glutaraldehyde. A portion of the fascia was sent for culture at the time of construction of the valve and again at the time of implantation of the fixed valve in the recipient.

Five Holstein calves and one sheep were used for implantation of the valves in the mitral position. At the time of implantation all valves were sterile to culture. The valves were noted to be very stiff and nonpliable. Two calves survived for two months. The other animals died within 24 hours from pulmonary edema. Postmortem examination showed the valves to be rigid, but not incompetent. The two calves that survived for two months also died from pulmonary edema. The valves were rigid and partially overgrown with neo-intima.

In vitro flow determinations across the valves showed them to be stenotic and nonpliable. The rigidity of the valves in the two month survivors demonstrated that the fixed fascia did not become more pliable after implantation. It was, therefore, concluded that glutaraldehyde-fixed facia lata is not a suitable material for use in constructing tissue valve prostheses.

Proposed Course: Paper being prepared for publication.

Serial No. NHLI-66
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of positive pressure ventilation on pulmonary vascular resistance

Previous Serial No.: None

Principal Investigators: Edward M. Mullin, M. D.
Robert E. Sloane, M. D.
Thomas Q. Winter, M. D.
Robert L. Reis, M. D.

Project Description: It has been a clinical impression that positive pressure ventilation with a volume controlled respirator can augment cardiac output and stabilize the hemodynamic state in the immediate postoperative period. This effect seems most pronounced in patients with elevated pulmonary vascular resistance secondary to long standing mitral valvular disease. To document and quantitate this effect, patients undergoing mitral valve replacement or open commissurotomy have been studied in the early postoperative period. Hemodynamic assessements with PVR measurements were recorded using first a volume respirator (Engstrom), and then during spontaneous ventilation via the Briggs adapter. Arterial blood gases were obtained with every intervention, and the patient's clinical status noted. Measurements were repeated during isuprel infusion.

Results: There is a statistically significant change in PVR (+60%) in switching from the Engstrom to Briggs adaptor, which was slightly modified by isuprel. The pH, pCO_2, pO_2 were relatively constant but cardiac output appears to decrease. Statistical analysis of these parameters is in progress. Preliminary data indicate that there is a beneficial effect from the use of positive pressure ventilation in the postoperative period.

Proposed Course: The manuscript is in process of preparation and will be submitted for publication.

Serial No. NHLI-67(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of the operative treatment of mitral stenosis

Previous Serial No.: None

Principal Investigators: Edward M. Mullin, Jr., M. D.
D. Luke Glancy, M. D.
Andrew G. Morrow, M. D.

Other Investigators: Lawrence Higgs, M. D.
Stephen Epstein, M. D.

Cooperating Unit: Cardiology Branch, NHLI

Project description: A study of closed mitral commissurotomies (CMC), open mitral commissurotomies (OMC), and valve replacements (MVR) for mitral stenosis performed from 1964 through 1969 was undertaken, to compare clinical status and catheterization findings. Records were reviewed for age, sex, rhythm, clinical classification, evidence of emboli, mitral calcification, previous and subsequent operations, and associated heart murmurs. Operative notes were reviewed for presence of LA thrombus, attempts at CMC or OMC, and mitral regurgitation incurred. Postoperative followup involved recording emboli, cause of death if any, and calls or letters to all patients not seen within the past year. Pre- and postoperative catheterization data were analyzed for LA, RV systolic, LA→LV gradient, CI, and MVA.

Results: The patients included 53 CMC, 20 OMC and 51 MVR. As expected the MVR patients represented an older group with more advanced valvular disease, a higher percentage of atrial fibrillation, equal sex distribution, more LA thrombi, and a higher operative mortality. There was no mortality in the CMC group, though four patients required subsequent operations. Two patients undergoing OMC succumbed (10%). Comparison of the catheterization data reveals that the best results were obtained in CMC and indicates that this is a satisfactory and safe operation in selected patients.

Proposed Course: A manuscript is in process and will be submitted for publication.

Serial No. NHLI-68(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Closure of atrial septal defect with a perforated patch

Previous Serial No.: None

Principal Investigators: Edward M. Mullin, M. D.
Robert L. Reis, M. D.

Project Description: The hemodynamic effects of patch closure of atrial septal defect have been well described, and the operation itself has become a relatively routine procedure. In a small group of adults, however, preoperative cardiac catheterization has suggested the possibility of left-heart decompensation with complete closure of an ASD. Two such patients have recently undergone operative repair of ASD using a perforated Teflon patch, to allow residual left-to-right shunting and to prevent sudden cardiac failure. The pre- and postoperative course, the catheterization data, and operative techniques have been reviewed and form the content of this project.

Results: In the first patient (K.D.), careful intraoperative pressure recordings as the ASD was closed are complimented by pre-and postoperative catheterizations. In the second (M.W.) postoperative data are pending though preoperative assessment was thorough.

Both patients have had long and complicated postoperative courses with evidence of LV failure. The successful use of a perforated interatrial patch to allow left heart decompression is a reportable technique and bears further investigation.

Proposed course: A manuscript is in progress and will be submitted for publication.

Serial No. NHLI-69(c)
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Augmentation of myocardial contractility with coronary bypass grafts

Previous Serial No.: None

Principal Investigators: Edward M. Mullin, M. D.
Robert E. Sloane, M. D.
Robert L. Reis, M. D.
Kenneth Kempner, M. A.

Cooperating Unite: Computer Systems Laboratory, DCRT

Project Description: Coronary bypass vein grafts are presently being performed in patients with coronary atherosclerosis and angina with the rationale that increased coronary blood flow beyond the point of obstruction will result in improved coronary perfusion. This improved perfusion should reflect itself in augmented contractility of the ischemic myocardium. The present study was designed as an intraoperative assessment of LV contractility by means of computer analysis of high fidelity LV pressure recordings. The contractile state was quantified by using V_{max} based on a graph plot of dp/dt/P against P (either total or derived).

Recordings of LV and Ao pressure were made after the vein grafts were constructed, and the patient successfully separated from bypass. Contractility was then measured with the graft (or grafts) open or occluded for 3-5 minutes. Where possible, this sequence was performed in duplicate. Changes in contractile state were correlated with vein graft flow as measured by individual flow probes.

Results: Although only a small number of observations (6) have been made, there appears to be a correlation between vein graft flow and changes in contractile state of the left ventricle. In cases where flow was minimal, there was also a minimal change in V_{max}. In one patient with substantial graft flow, there was a significant (30%) augmentation of the contractile state. This method of analysis of the effect of coronary vein grafts has merit and warrants further investigation.

Proposed course: Continued application of intraoperative contractile assessment of the effects of coronary bypass grafts.

Serial No. NHLI-70
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The effect of protamine sulfate on myocardial contractility in the intact dog

Previous Serial No.: None

Principal Investigators: Gordon N. Olinger, M. D.
Robert E. Sloane, M. D.
Edward B. Stinson, M. D.
Andrew G. Morrow, M. D.

Project description: Normal adult dogs were instrumented with aortic flow probes and solid state aortic and left ventricular pressure transducers and were studied in the intact state several days after recovery from operation. Two dogs also had regional cardiac denervation. Animals were studied while sedated and determinations made on-line (utilizing analog computation of left ventricular dp/dt/P plotted against P, i.e., contractile element velocity) with use of V_{max} as an index of myocardial contractility. The effect of therapeutic dosage of protamine sulfate given intravenously was evaluated in each dog both in the normal resting state, and, during another study, after beta-adrenergic blockade with propranolol. Preliminary data have shown no effects on contractility in these preparations. A small number of dogs similarly instrumented were subjected to standard periods of cardiopulmonary bypass and protamine sulfate evaluated following cessation of bypass. Small decrements in contractility have been observed under these circumstances. Protamine sulfate has shown no effect in the normal dog made acutely hypertensive with neosynephrine.

These preliminary data indicate that protamine sulfate does not have a direct negative inotropic effect on the normal canine myocardium. Its action on the nyocardium following cardiopulmonary bypass indicates some vasoactive influence which remains to be elucidated. The mechanisms of this action are currently under evaluation.

Proposed Course: Further elucidation of the mechanism of action of protamine sulfate on the peripheral vascular system. To be submitted for publication.

Serial No. NHLI-71
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of the effects of corticosteroids on myocardial contractility

Previous Serial No.: None

Principal Investigators: Paul L. Tecklenberg, M. D.
Edward B. Stinson, M. D.
Andrew G. Morrow, M. D.

Project Description: Eleven normal, nine chronic cardiac denervated (four after beta-blockade with propranolol), and five acutely adrenalectomized and cardiac denervated dogs were studied on cardiopulmonary bypass, at constant coronary blood flow, by means of an isovolumic left heart preparation. Methyl-prednisolone (MP), 15-30 mg./Kg., was given IV and changes in myocardial contractility were assessed by measurement of left ventricular peak pressure (LVP), LV dp/dt, and LV contractile element velocity (max V).

Results: In normal dogs MP caused significant enhancement of myocardial contractility (LVP + 21%, LV dp/dt + 27%, max V + 22%, $P < .01$ in all). Chronic cardiac denervated animals showed similar effects, but with significantly greater responses than normal dogs in LVP and LV dp/dt ($P < .01$). These changes were greatly attenuated or abolished in beta-blocked-denervated, and in acutely adrenalectomized and denervated dogs.

The results of this study confirm an acute positive inotropic effect of MP in vivo, but suggest that this influence is mediated indirectly by the release and/or potentiation of endogenous catecholamines.

Proposed Course: Manuscript to be submitted for publication.

Serial No. NHLI-72
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanisms of the pulmonary vascular response to hypovolemic shock

Previous Serial No.: None

Principal Investigators: Frederick H. Levine, M. D.
Robert L. Reis, M. D.

Other Investigators: Jefferson F. Hollingsworth, M. D.
David M. Conkle, M. D.

Project Description: Increased pulmonary vascular resistance (PVR) which occurs with hypovolemic shock (S) is thought to be related to both neural and humoral factors. The pulmonary and systemic circulations were separately perfused by two pump oxygenator systems. Neural pathways were intact but humoral isolation of the circulations was confirmed. A pulsatile pump was employed in the systemic circulation and stroke volume was adjusted so that mean arterial pressure was 100 mm. Hg. Systemic and pulmonary arterial pO_2 and pCO_2 were maintained physiologic. Pulmonary blood flow, systemic blood flow, pulmonary arterial, left atrial, and systemic arterial pressures were continuously recorded. PVR was assessed before and after (S) by resistance-flow curves constructed by increasing pulmonary flow from 50-200 cc./Kg./min. (S) was produced by decreasing stroke volume until mean arterial pressure was 30 mm. Hg. Twenty experiments were performed on ten dogs. PVR was unchanged when assessed 5 minutes after (S) was induced. One liter of blood (physiologic pH) obtained from the systemic circulation 10 minutes after (S) was added to the pulmonary circulation; PVR increased from 561 ± 70 (dynes-sed-cm^{-5}) to 748 ± 90 ($p < .05$). Systemic acidosis 9pH $7.14 \pm .06$) developed after 30-45 minutes of (S) but the PVR remained unchanged. When the pH of the pulmonary blood was decreased ($7.08 \pm .08$) by lactic acid infusion PVR increased from 521 ± 104 to 794 ± 130 ($p < .05$).

Thus neither systemic hypotension (baroreceptors) nor metabolic acidosis in the systemic circulation (chemoreceptors) produced reflex changes in PVR. Increased PVR in (S) results from the effects of humoral factors on the lung.

Proposed Course: Project completed - abstract submitted for publication.

Serial No. NHLI-73
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Hemodynamic effects of bretylium tosylate in the intact dog

Previous Serial No.: None

Principal Investigators: Robert E. Sloane, M. D.
Gordon N. Olinger, M. D.
Edward B. Stinson, M. D.
Andrew G. Morrow, M. D.

Project Description: Controlled studies of the inotropic effect of bretylium tosylate have been done in vitro. However, data obtained from intact mammals is inconclusive. Miniature solid state pressure transducers were implanted in the left ventricle and proximal aorta of dogs. Aortic flow probes and atrial pacing wires were placed as well. From 3 to 7 days postoperatively, the animals were studied in intact state. Myocardial contractility was determined by extrapolating to V_{max} the linear isovolumic portion of the vector loop produced by dp/dt/P-LVED displayed against P-LVED on an oscilloscope. Control V_{max}, dp/dt, LVP, LVEDP, AoP, and cardiac output were compared before and after administration of bretylium tosylate 5 mg./Kg.

Results: The study is at present in progress so that results are preliminary. A biphasic rise in V_{max} was noted in the order of 10-40%; dp/dt also rose 10-100%. LVP rose, LVED fell, mean AoP increased and C.O. did not change significantly. The inotropic effect of the drug was confirmed. Prior administration of propranolol 0.5 mg./Kg. completely abolished the biphasic inotropic action of bretylium. Further studies of the mechanism of action are in progress.

Serial No. NHLI-74
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The use of isobutyl cyanoacrylate in microvascular anastomosis

Previous Serial No.: NHLI-87

Principal Investigators: Sherman G. Souther, M. D.
Sidney Levitsky, M. D.
Andrew G. Morrow, M. D.

Other Investigator: William C. Roberts, M. D.

Cooperating Unit: Pathology Section, Cardiology Branch, NHLI

Project Description: The infrarenal abdominal aorta in albino rats weighing 120 to 480 grams was divided between occluding clamps, and stay sutures of 8-0 and 9-0 nylon were placed. Isobutyl cyanoacrylate was applied to the approximated edges of the divided vessel, and the clamps were removed. The animals were sacrificed at intervals varying from 24 hours to 6 weeks, and sections of the anastomoses were obtained for histologic study. This group of rats was compared to a control group in which anastomoses performed with a continuous suture of 8-0 or 9-0 nylon.

Results: Five of 28 anastomoses in the isobutyl cyanoacrylate group became occluded, and 1 of 24 anastomoses in the suture group became occluded. Comparison of sections through patent anastomoses revealed a more marked and prolonged acute and chronic inflammatory response in the vessels in the isobutyl cyanoacrylate group than in the suture group. In addition, degeneration of the media and deposition of calcium occurred in the vessels in the isobutyl cyanoacrylate group.

Proposed Course: A manuscript has been prepared from these data and has been submitted for publication. The project is completed.

Serial No. NHLI-75
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Prolongation of cardiac allografts in the rat by alloantisera

Previous Serial No.: None

Principal Investigators: Sherman G. Souther, M. D.
Edward B. Stinson, M. D.
Andrew G. Morrow, M. D.

Project description: Survival of heterotopic (abdominal) heart allografts was studied in inbred rats. Nine control Lewis rats (L) rejected (loss of EKG activity) Brown Norway (BN) grafts in 6.4 ± 0.7 (S.D.) days. Thirteen L rejected hybrid (LBN) grafts in 14 ± 3.4 days. IV administration of 10^7 BN or LBN bone marrow cells to seven L and spleen cells to five L seven days preoperatively caused significant prolongation of BN grafts to 11.7 ± 3.3 and 9.6 ± 2.1 days respectively ($p < .05$). Administration of BN bone marrow or spleen cells to 10 L did not cause significant prolongation of LBN graft survival. Serum obtained from L seven days after injection with either 10^7BN or LBN spleen cells was given IV intraoperatively and for nine days postoperatively (total 8 ml.) to each of four L with BN grafts, and prolonged survival to 11.0 ± 2.4 days ($p < .05$). Control sera did not prolong graft survival.

Alloantiserum prolongs survival of cardiac allografts in the rat, although less dramatically than previously shown for renal grafts.

Proposed course: The project is completed and a manuscript will be prepared.

Serial No. NHLI-76
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mixed leucocyte reaction (MLR) as an assay for the presence of enhancing alloantiserum

Previous Serial No.: None

Principal Investigators: Sherman G. Souther, M. D.
Edward B. Stinson, M. D.
Robert O. Gordon, M. D.
Andrew G. Morrow, M. D.

Other Investigator: Joost J. Oppenheim, M. D.

Cooperating Unit: Laboratory of Microbiology, NIDR

Project description: Alloantisera that enhance survival of heterotopic (abdominal) heart allografts in inbred rats were tested in MLR. Lewis (L) rats administered 10^7 Brown Norway (BN) rat bone marrow or spleen cells intravenously (IV) 7 days prior to transplantation exhibited significant prolongation of BN graft survival. Passive administration to normal L rats of serum obtained from L rats 7 days after such IV immunization similarly prdonged BN graft survival. The unidirectional MLR of normal or presensitized L with irradiated BN spleen cells was also suppressed by this immune serum as compared to MLR's containing normal L serum. However, washed splenic leucocytes from these presensitized L rats when incubated in normal L serum showed increased proliferative activity to irradiated BN spleen cells. Since these L cells after removal of "enhancing" serum were very active in the MLR, this suggests that enhancement rather than central tolerance was the mechanism of graft prolongation. Twenty percent alloantiserum decreased the MLR of unsensitized L with irradiated BN as much as 6-fold. Decreasing degrees of suppression were demonstrated with 10% and 5% antiserum, and none with 2.5%. Alloantiserum suppression of MLR remained demonstrable in the absence of hemolytic complement. These suppressive antisera have no cytotoxic effects in microcytotoxicity assays. Immunologic specificity was demonstrated because "enhancing" serum failed to suppress the MLR of L and irradiated ACI rat leucocytes. Thus, inhibition of MLR can be used as an assay for the presence of "enhancing" alloantiserum.

Proposed course: The immune serum is being evaluated for specific antibody and the importance of the complement fixing fragment.

Serial No. NHLI-77
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immediate *in vitro* leukocyte DNA synthesis: An early indicator of heart allograft rejection

Previous Serial No.: None

Principal Investigators: Sherman G. Souther, M. D.
Edward B. Stinson, M. D.
Robert O. Gordon, M. D.
Andrew G. Morrow, M. D.

Other Investigator: Joost J. Oppenheim

Cooperating Unit: Laboratory of Microbiology, NIDR

Project description: The *in vitro* DNA synthesis by splenic leukocytes from Lewis (L) rats bearing heterotopic (abdominal) Brown Norway (BN) rat heart allografts was studied. Allografts in nine L rats were rejected (loss of EKG activity) in 6.4 ± 0.7 (S.D.) days. Twelve L rats were sacrificed at days 1-6 (2 each day) after grafting; the allografts were excised for pathologic study, and the spleens were removed for study of leukocyte DNA synthesis. One μci of ^{3}H-thymidine was added to a suspension of 3×10^{6} L leukocytes in 1.5 ml. RPMI 1640 culture medium supplemented with 10% fetal calf serum. After two hours of incubation at 37° C, the cells were washed with isotonic saline and treated with 5% TCA at 4° C. The acid precipitable radioactivity was determined. Mean counts per minute (cpm) of 12 normal L rat spleen cell suspensions was 527 ± 165 (S.D.) (range 204-734). Mean cpm of animals with allografts were increased above normal L as follows: day 2, 1.3x; day 3, 2.0x; day 4, 2.4x; day 5, 7.2x, and day 6, 7.5x. By day 3 the increase in DNA synthesis was significantly elevated above normal, and there was no overlap with the range of normal L rats. Sham operated animals showed no increase in lymphocyte activation. Changes in the status of the grafts determined by palpation and EKG were inconsistent and were invariably preceded by increased *in vitro* leukocyte DNA synthesis by at least 48 hours.

Proposed Course: These studies will be continued utilizing peripheral blood leukocytes in dogs bearing orthotopic cardiac grafts.

Serial No. NHLI-78
1. Clinic of Surgery
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Creation of aorto-pulmonary shunts with cyanoacrylate tissue adhesive

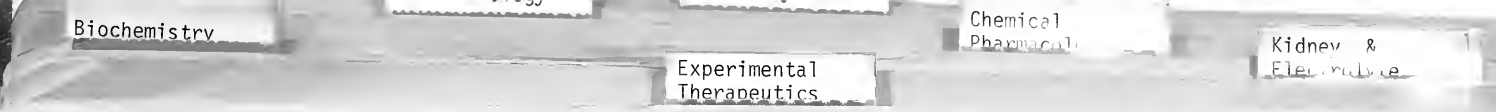
Previous Serial No.: None

Principal Investigators: Sherman G. Souther, M. D.
Edward B. Stinson, M. D.
Andrew G. Morrow, M. D.

Project description: A rapid method of creating aorto-pulmonary shunts was devised. Through a left thoracotomy in dogs, the common adventitia between the aorta and main pulmonary artery was opened, and the aorta and pulmonary artery were approximated with isobutyl cyanoacrylate or fluoroalkyl cyanoacrylate. Through a purse-string suture on the opposite wall of the pulmonary artery, a large needle was inserted into the pulmonary artery and then into the aorta. The needle was removed, and a Fonkalsrud septectomy punch was inserted into the aorta through the tract made by the needle. The wall of the aorta and pulmonary artery in the area approximated with the tissue adhesive was engaged with the punch and a portion was removed. The purse-string suture was tied. A good thrill in the pulmonary artery indicated a satisfactory shunt.

Results: Thirty-two dogs, 18 adults and 14 puppies, had shunts performed. A 5 mm. diameter punch was used in adults, and a 3 mm. punch was used in puppies. Four adults and one puppy died from intraoperative hemorrhage; one adult and one puppy died from late postoperative hemorrhage. In five adult dogs, the operation was done without dissection of the aorta and pulmonary artery and without application of the glue. These animals were considered a control group. Three months after operation, two adult dogs with continuous murmurs were reoperated, and 2 mm. and 3 mm. shunts were closed using total cardiopulmonary bypass. These dogs survived without difficulty. The remainder of the 11 adults were sacrificed three months after operation. Two dogs had continuous murmurs, and each had a 2 mm. shunt at autopsy. The remaining nine dogs had no murmur, and at autopsy the shunt was completely closed.

Proposed course: The puppies will be followed for one year to evaluate the late results of operation.


Biochemistry
Experimental
Therapeutics
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ANNUAL REPORT OF THE
EXPERIMENTAL THERAPEUTICS BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

BIOCHEMICAL PHARMACOLOGY

I. Biochemistry of Neurohumoral Amines

Enzymes of the Pineal Gland. A protein fraction from rat liver was found to stimulate tryptophan hydroxylase isolated from beef pineal. Since the fraction appears identical to liver phenylalanine hydroxylase, we believe that these two hydroxylating enzymes are similar and that substrate specificity may be determined by various subunit combinations. Hydroxyindole O-methyltransferase (HOMT) has been purified from beef pineals and shown to have a molecular weight of about 78,000, consisting of 2 identical subunits of 39,000. This enzyme is a major protein of the pineal representing 4% of the total soluble proteins in this gland. HOMT exists in 2 higher molecular weight forms and in 2 differently charged species. It is possible that conversion of one form to another may result in changes in enzyme activity noted during diurnal variations. Protein kinases may play a key role in control of such diurnal rhythms acting either at a transcriptional level (histone phosphorylation) or by modifying enzymes directly. Bovine pineals were found to contain active protein kinases which are strongly stimulated by cyclic AMP. At least two of these kinases have been partially purified.

Serotonin: Chronic transection (5 weeks) of spinal cord in cats results in an 80% loss of tryptophan hydroxylase and serotonin content. Since all the descending serotonergic fibers degenerate, residual serotonin and synthetic enzyme suggest the presence of serotonergic interneurones. Other studies in cats demonstrate that administration of 5-hydroxytryptophan enhances monosynaptic reflexes and that tricyclic antidepressant drugs act synergistically to greatly enhance the effect. This finding has relevance to clinical situations such as hypotonia in Down's syndrome.

Other studies reveal the following: 1) serotonin synthetic rates and tryptophan hydroxylase levels are not altered in morphine addicted mice, contrary to reports from other laboratories; 2) there is no change in the level of tryptophan hydroxylase during REM sleep deprivation in rats, although others report an increase in serotonin turnover; and 3) using a system for continuous monitoring of gastric secretion in the rat, an inhibitory effect of serotonin (i.v.) on histamine stimulated secretion was observed while 5-hydroxytryptophan had no effect.

Histamine: The development of new methods of assay for histamine and its metabolizing enzyme, histaminase, has permitted more definitive studies on this compound. The gastrointestinal tract contains the highest amount of histaminase in the rat, although appreciable amounts exist in the adrenals, pancreas and thymus. Histaminase activity is increased in the thymus of spontaneously hypertensive (SH) rats compared to controls. The rise in plasma histaminase following dosing with heparin was accompanied by a severe

depletion in the intestinal content of the enzyme. The plasma level of histaminase returned to normal within 5 hours and the intestinal level was restored within 10 hours.

The possible role of histamine in inflammation and swelling was examined using the heat injured paw of the rat. Heat injury caused a 70% depletion of histamine in paw skin, substantial swelling and a 5 to 10-fold increase in the histamine content of paw exudate. Prior depletion with the histamine liberator 48/80 partially blocked the effects. Compound 48/80 causes the release of histamine from mast cells by an energy requiring reaction. The release is strongly inhibited in vitro by dibutyryl cyclic AMP and a variety of compounds which inhibit phosphodiesterase. It appears that cyclic AMP is involved in release of histamine from mast cells.

Catecholamines: The brainstem levels of norepinephrine in spontaneously hypertensive (SH) rats are 20 to 30% less than those of normal rats. It was found that the aromatic L amino acid decarboxylase in the brainstem was decreased by 50% possibly accounting for the lower norepinephrine content. A number of studies were done using catecholamine precursors to replete the brainstem levels. A significant inverse correlation between blood pressure and brainstem norepinephrine was observed. Peripheral administration of 6-hydroxydopamine which destroys a large percentage of the peripheral sympathetic nerves was not effective in reducing the blood pressure in the hypertensive animals. The plasma renin activity of SH rats was found to be distinctly elevated. This renin activity appeared to be of extrarenal origin, but no direct correlation between this enzyme and catecholamines has been established.

II. Metalloproteins

The primary amino acid sequence of the iron-sulfur protein, rubredoxin, has been largely completed. The points of major interest are the finding of N-formylmethionine at the N terminus and the positioning of the cysteinyl residues. The sulfhydryl groups of these 4 cysteinyl residues form the ligands for the iron atom and are arranged in a tetrahedron. Evidence for this type of binding has been obtained by near infrared absorption and circular dichroism and by Laser-Raman spectroscopy. There does appear to be a distortion in the tetrahedral symmetry as evidenced by highly dichroic single crystal absorption spectra and splitting of the $d \rightarrow d$ transition bands in the near infrared. These observations have been confirmed by x-ray crystallographic analysis.

The techniques for measuring absorption and circular dichroism of protein solutions in the near infrared have been applied to plant ferredoxin, and adrenodoxin. At least one of the two iron atoms in each of these proteins is bound in a tetrahedral configuration. Spectral analysis also indicates that there is significant interaction between the two iron atoms in each molecule. Based on these observations and other reported physical studies, a model for the active center of these two important iron-sulfur proteins has been proposed.

PHYSIOLOGICAL CHEMISTRY

I. Factor XIII and Fibrin Crosslinking

Fibrin clots from normal plasma contain approximately 6 moles ε-(γ-glutamyl)lysine crosslink per mole fibrin, whereas clots from individuals with Factor XIII deficiency contain little or none of this crosslink (0.02-0.64 mole/mole). Thus, recurrent bruising and bleeding, and abnormal wound healing in these cases may be due to the lack of ε-(γ-glutamyl)lysine formation.

II. Kinin System

The purification of human and rat urinary kallikreins has permitted the development of an improved assay for urinary kallikrein, which has been applied clinically. In SH rats urinary kallikrein was significantly elevated (at 11 and 16 weeks) above that in control animals. Significant progress has been made in the purification of what may be a specific activator of prekallikrein which the data indicate arises from inactive Hageman factor. Antibodies to human and rat urinary kallikrein have been prepared. Both block action of the enzyme on kininogen but ester hydrolysis is not affected. The principle of affinity chromatography has been used in the purification of human prekallikrein activator, human kallikrein and human kininogens.

III. New Biologically Active Peptides

The peptide nature of villikinin has been more firmly established with studies on material of bovine origin. As with canine material, bovine villikinin is a low molecular weight substance which can be purified on cation exchange resins. It is also inactivated by Pronase, papain and chymotrypsin and not by trypsin. In confirmation of work elsewhere a pressor substance was found in the plasma of dogs following bilateral renal artery ligation. The biologic spectrum of activity is similar to that of angiotensin I, but not angiotensin II. Boiling the plasma abolishes activity suggesting an angiotensin I-protein complex. The bald-faced hornet venom sac was shown to contain 1-5 mg of serotonin and histamine, as well as hypotensive substance which may also be a peptide. Eight synthetic analogues of ranatensin (undecapeptide) were tested for hypotensive and uterine stimulant activity. The c-terminal nona and deca peptides were fully active, while Val^6-Ala^7 ranatensin was inactive on blood pressure and the uterus.

IV. Biochemical Analysis

Droplet countercurrent chromatography (DCCC, earlier described by us) has been further developed with the construction of an even simpler unit consisting of only teflon tubing. DCCC has been used for the separation of peptides, proteins, and ribonucleic acids. Reproducibility is excellent, elution volumes are accurately predicted from the partition coefficient and recovery is quantitative with as little as 1 μg of material. Another form of countercurrent chromatography, gyration locular countercurrent chromatography (invented in LTD, NHLI), has been evaluated and found to have higher resolving power and speed of analysis than DCCC, but lower capacity and more

complexity in execution.

The gas chromatographic (GC) method for the analysis of phenylthiohydantoin derivative of amino acids has been improved by: development of a new blend of stationary phases that gives better isolation and faster analysis; the use of helium as carrier gas; and high temperature conditioning of the columns. Methylthiohydantoins were also studied and separated with greater resolution than hitherto obtained. Chemical ionization mass spectrometry (CIMS, performed by LC, NHLI) was compared to GC for assay of phenylthiohydantoins; while GC is simpler, the CIMS method has the potential of greater sensitivity and speed of analysis when used with a computer and automatic sample applicator.

EXPERIMENTAL MEDICINE

I. Histamine

A specific radioassay for histamine in urine has been developed. Normal histamine excretion averaged 11 µg/24 hr (n=16) with a range of 3 to 40; these values are lower than that found with less specific (e.g. fluorescence) assays. β-^{3}H-histamine has been prepared enzymatically and its fate studied in 6 patients. The β-^{3}H label permits distinguishing between the activity of histaminase (diamine oxidase) which releases tritiated water and the activity of monoamine oxidase which leads to formation of tritiated acidic metabolites. Histaminase activity accounted for the metabolism of only 5 to 8% of the injected histamine. Histamine-N-methyl-transferase and monoamine oxidase are the more important enzymes in histamine metabolism, and not histaminase as had been thought previously. We have found abnormally high levels of serum histaminase in 15 of 23 patients with medullary carcinoma of the thyroid. High levels of histaminase activity are also present invariably in tumor tissue. Thus, serum histaminase activity serves as a useful diagnostic tool and tissue histaminase activity serves as a tumor marker. Plasma histaminase activity increases markedly after small doses of intravenous heparin, the response being similar to that of plasma lipoprotein lipase activity. Four patients with Type I hyperlipoproteinemia and one with Type IV had subnormal responses of serum histaminase activity to both low (10 units heparin/kg) and high (75 units/kg) doses of heparin. The relationship of histaminase to lipolysis, if any, is unknown.

II. Kallikrein

Kallikrein is an enzyme which controls the production of bradykinin, the most potent vasodilator known. An improved esterolytic assay has been used to study extensively the excretion of urinary kallikrein in man. Significant differences from normal were found in hypertensive subjects, e.g. a mean of 3.8 enzyme units (per 24 hr) in male hypertensives compared to 9.0 in normal men. Two hypertensive subjects had no detectable urinary kallikrein; a possible genetic relationship is being studied.

III. Cardiovascular Drugs

MK-266 (a benzyl ether of metaraminol) has been administered to 7 patients with essential hypertension. At doses of 30 to 800 mg/day there were slight to moderate decreases in blood pressure, most marked usually on the early morning standing pressure. Three patients had significant changes in SGOT and SGPT levels which were probably drug related. The drug is not a good source of metaraminol since only 1% of the administered drug could be recovered as metaraminol in the urine.

A technique has been developed for studying the reactivity of small strips of human temporal artery in vitro. Tissue has been obtained from seven normotensive neurosurgical patients. Dose-response curves, and the agonist-antagonist values for alpha adrenergic receptors were found to be comparable to those of animal blood vessels. This technique holds great promise for precise evaluation of vascular responsiveness in patients (hypertension, etc.).

IV. Collagen Metabolism

Seventy per cent of 54 patients with hepatoma were found to have abnormally high values of serum protocollagen proline hydroxylase (PPH) activity. This is comparable to the occurrence of alpha-fetoprotein in hepatoma. Thus, serum PPH activity serves as a useful diagnostic test for hepatoma and we are now studying it serially to evaluate the effects of therapy. Elevated serum PPH activity also seems to indicate liver cell injury and has been found to rise acutely in both man and rabbits 24 hours after exposure to fluorinated hydrocarbon anesthetics. In tissues PPH activity is a good indicator of the rate of collagen synthesis. We have found that PPH activity is markedly elevated in the skin of patients with scleroderma and in keloid and hypertrophic scars. These findings help to explain the excessive collagen found in these conditions and provide more insight into pathogenesis and treatment.

NEUROENDOCRINOLOGY

I. Taste

We have hypothesized that some form of chemical sieving controls the non-specific portion of pre-neural taste events. Experiments demonstrating inhibitory effects of thiol-containing drugs and amino acids support this concept. Locally applied proteases affect all taste qualities and suggest that these enzymes are effective on protein of the taste bud exposed to the oral environment. We suppose this protein is part of the membrane of the predominant cells of the taste bud, the type I receptor cell. There is also a specific portion of the pre-neural events of taste which relate to each taste quality. The specific taste events are most probably involved with the binding of tastant to the receptor membrane.

We have described an equation by which taste phenomena occur in normal man and in patients with various abnormalities of adrenal cortical function. This equation is:

$$I/I_{max} = \frac{K\,(T)^2}{K\,(T)^2 + 1}$$

where I is the intensity of tastant at concentration (T), I_{max}, the maximum intensity at highest (T) and K is a constant. The model which is described by this equation would explain why intensity functions in taste reach a maximum at high tastant concentrations; taste molecules would complex with all available receptor molecules and introduction of more tastant could not form more complexes. Deficiency and excesses of carbohydrate-active steroids in man may affect the pre-neural events of taste by directly affecting the binding constants by which tastant-receptor molecule complexes occur or by affecting the stoichiometry of binding. Our data demonstrate that excessive and inadequate amounts of these steroids decrease the binding constants for all taste qualities because the intensity curves are all shifted to higher concentrations. Apparently there is a concentration of steroid at which intensity is maximal for a given tastant concentration and either an excess or a deficiency of steroid lowers the intensity.

We have placed the entire problem of taste acuity within the framework of medical practice. The prevalence of the disease, Idiopathic Hypogeusia, which we have recently described, indicates the need for an awareness and an understanding of the loss of taste. Results of a single blind study indicate that oral administration of zinc is beneficial in patients with this disorder although the mechanism is not yet known. Injection of Zn^{65} in rats demonstrated that the tongue was one of the most active tissues in accumulating label, behind bone and liver. Correlating these studies with those previously noted in which zinc was found in the epithelial layer of papillae by laser microprobe spectroscopy suggests that taste bud bearing papillae and perhaps even taste buds themselves may avidly take up zinc.

II. Olfaction

We have limited the role of vitamin A in olfaction to one which involves retinol binding protein (RBP) and vitamin A alcohol, separated abnormalities of vision from olfaction in hepatitis and clarified the interrelationship between olfactory function and gonadal function in lower mammals. An exhaustive report (5,000 citations) on the molecular basis of olfaction has been prepared for the Office of Naval Research during this past year; the requirements which an adequate molecular theory of olfaction must satisfy have been established.

III. Trace Metal Metabolism

1. Metals and Hormones. We have found that the Δ^5-3 hydroxysteroid-dehydrogenase-isomerase step in steroidogenesis in rat and cat requires copper. Tissue metal concentrations are dependent on endogenous adrenal steroids. The liver appears to be an important source of metals which are mobilized by increased amounts of circulating adrenocorticosteroids. Serum concentrations of copper and zinc are influenced by both estrogen and progesterone, but not by LH or FSH. It is interesting that intrauterine devices coated with copper are more effective in controlling fertility than the same devices coated with Teflon. We have established that serum concentrations of copper (also ceruloplasmin) and zinc exhibit circadian changes parallel in time to secretion of cortisol; similar changes were observed for urinary copper but not zinc. Interestingly, these changes are not abolished

by blocking endogenous adrenocortical or ACTH secretion for 3 days.

2. Metal-Protein Interactions. Metals in urine are not "free" but rather appear as ligands with relatively small molecular weight peptides or proteins. Two distinct metal containing proteins have been identified in dog kidney. One (MW 34,000) contains equal amounts of copper and zinc and is probably cytocuprein. The other (MW 10,000) corresponds to metallothionein in size but contains only copper, without zinc or cadmium. Kidney metallothionein may differ significantly in different animal species.

3. Metal-Neurotransmitter Interactions. Metals are generally inhibitory of the uptake of norepinephrine (NE) and choline (Ch) by brain synaptosomes and for the activity of both Na-K activated and Mg activated ATPase activity. The specific nature of this inhibition has been systematically investigated and related to the nature of the metal-neurotransmitter complex. However, two metals, Mn and Sn, appear to enhance NE uptake. Sn acts to enhance Na-K ATPase activity specifically (without an effect on Mg-ATPase activity) and thereby the uptake of NE by brain synaptosomes. These studies suggest a physiological role for Sn.

IV. Steroid Hormones and Neural Function

Steroid hormones are known to influence sensory and central nervous function. NE and Ch uptake by brain synaptosomes is markedly inhibited by carbohydrate-active steroids, apparently via inhibition of Na-K activated ATPase. No effect of these steroids was observed on Na or K currents of the isolated squid nerve indicating their effect on neural function is on metabolism of nerve (i.e. maintaining membrane potential); they also appear to affect synaptic delay. In addition, these steroids appear to operate through effects on myelin.

Serial No. NHLI-79(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Urinary Excretion of Kallikrein

Principal Investigator: Harry S. Margolius, Ph. D., M.D.

Other Investigators: Ronald Geller, Ph. D., Wybren DeJong, M.D., Vida Beaven, Ph. D. and John Pisano, Ph. D.

Cooperating Units: Normal Volunteer Office

Project Description:

Objectives: To identify the magnitude of urinary excretion of kallikrein in normal volunteers, patients with various diseases, control and spontaneously hypertensive rats (SHR), and anesthetized dogs subjected to various manipulations and drugs.

Methods: Patients: 24 hour urine collections were obtained from 50 normal volunteers and a like number of subjects with various diseases. The amount of kallikrein excreted was measured in two ways: 1) using the method of Beaven, et al (Clin. Chim. Acta, In Press) which measures esterolytic capability of urine kallikrein and 2) using standard bioassay techniques. Recoveries of pure standard human urinary kallikrein were measured in each urine sample and ranged from 70-100%.

Rats: 24 hour urine collections were obtained from male Wistar control, Sprague-Dawley control and SHR. Urine was assayed for kallikrein esterase activity and kallikrein biologic activity. In addition, urine volume, body weight, urine protein excretion and blood pressure were also measured.

Dogs: The effects of acute unilateral renal artery constriction and angiotensin infusion on ureteral excretion of kallikrein were measured in 20-25 kgm dogs anesthetized with pentobarbital. Both bioassay and esterolytic activity were assessed, as well as arterial blood pressure, renal arterial blood flow and urine output.

Major Findings: Patients: The 24-hour urinary excretion of kallikrein ranged from 3.76 to 19.38 enzyme units (I.E.U. = 1 μmole Tolsylarginine methylester hydrolyzed/min) with a mean of 9.00 in 25 normal males. In 23 normal females the range was 2.43-19.67 E.U. with a mean of 6.66 E.U. In 11 male patients with essential hypertension, the values ranged from 0.00-8.91 with a mean of 3.78 E.U./24 hour. In 9 female patients with essential hypertention excretion ranged from 0.00-7.52 with a mean of 2.99 E.U./24 hours. In 8 patients with proven pheochromocytoma kallikrein excretion varied from 3.53-33.27 with a mean of 14.92 E.U./24 hours. No consistently abnormal

findings have been found, as yet, in patients with burns, carcinoid syndrome, hypoparathyroidism, or metastatic carcinoma. It is of interest that both the parents of a 27-year old white female with hypertension of unknown etiology, and a urine kallikrein excretion of 0.00 E.U./24 hours, also excrete abnormally low amounts of kallikrein in their urine.

Rats: Urine kallikrein excretion in control Wistar and spontaneously hypertensive rats (SHR) was examined in male animals at 7,11,16 and 25 weeks of age. At seven weeks, levels of urinary kallikrein were similar in both groups; significant increases appeared in SH rats at 11 weeks and were greater at 16 weeks but declined by 35 weeks. In all groups the SHR exhibit higher urinary excretion rates and higher blood pressures - while body weight, urinary protein excretion and urine volume are similar.

Dogs: We have been unable to demonstrate acute changes in kallikrein excretion rates to date. Neither unilateral renal artery constriction nor angiotensin infusion have produced significant or consistent changes in urinary kallikrein excretion rate or concentration.

Significance: The data indicate that urinary excretion of kallikrein, an enzyme which controls production of the most potent vasodilator substance known, bradykinin, is altered in hypertension in animals and man. This finding clearly separates a special group of patients with hypertension. This may provide a starting point for understanding the role of kinins in pathogenesis of, or compensatory responses to hypertension.

Proposed Course of Project: Man: Enlarge and clarify patient categories in which urinary kallikrein excretion is abnormal, and correlate plasma kallikrein levels and assess kinin metabolism in hypertension.

Rats: Parallel studies in SHR, renal and DOCA-salt hypertensive and control animals.

Publications: None

Serial No. NHLI-80(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Biological Role of Histamine: Histaminase Activity in Various Physiological and Pathological States.

Previous Serial Number: NHLI-162(c)

Principal Investigator: Stephen B. Baylin, M.D.

Other Investigators: Michael A. Beaven, Ph.D., Zdenka Horáková, Ph.D., Harry R. Keiser, M.D., and Albert Sjoerdsma, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: This project is a continuation of the clinical studies of histaminase activity in normal and disease states. The studies included: 1) further investigation of the elevated histaminase activity in serum and tissues of patients with medullary carcinoma of the thyroid; 2) study of the appearance of histaminase in plasma after heparin in normal individuals and in patients with hyperlipoproteinemia; and 3) evaluation of the effect of the histaminase inhibitor, aminoguanidine, on food consumption.

There are two conditions in normal individuals in which plasma or serum histaminase activity may be high; pregnancy and after the administration of heparin. Previous studies from this laboratory have shown that high serum activity may be present in some patients with medullary carcinoma of the thyroid, and that high histaminase activity is present in this tumor tissue. The relationship between histaminase and this malignancy has been further explored. Additional work is now being done concerning the dynamics of plasma histaminase activity increase after parenteral heparin. Since this phenomenon is not specific to histaminase, and other enzyme activities like lipoprotein lipase also increase, studies of plasma histaminase activity are being done in a disease state involving a deficiency of lipoprotein lipase, Type I hyperlipoproteinemia (Fredrickson classification).

Methods: The assay of histaminase activity, by the measurement of tritiated water formation upon deamination of β-^{3}H-histamine, was described in Project Report NHLI-162(c), 1969-70.

All subjects who participated in the studies were either patients or normal volunteers hospitalized in the Clinical Center, NIH. Serum samples from patients with medullary carcinoma of the thyroid have been donated by several investigators throughout the country, in particular, Dr. Kenneth Melvin, New

England Medical Center, Tufts University, Boston, Mass.

Major Findings: 1) Histaminase activity in medullary carcinoma of the thyroid. The previous observations of elevated serum histaminase activity in patients with medullary carcinoma of the thyroid have been extended. In 50 normal individuals the mean serum histaminase activity was 1.5 units/ml serum (1 unit = 1 μmole histamine deaminated/hr). Of the 23 patients with medullary carcinoma of the thyroid, 21 (91%) had serum histaminase activity greater than 2.4 units/ml (> 1 standard deviation from the normal mean) and 15 patients had serum histaminase activity greater than 3.5 units/ml (> 2 standard deviations from normal). In these 15 patients, serum histaminase activity ranged from 3.6 to 90 units/ml.

The histaminase activity of normal human tissues and of tumor tissues from patients with widely disseminated medullary carcinoma of the thyroid was examined. Only kidney and intestine had histaminase activity greater than 50 units/g tissue. In contrast, the presence of microscopic foci of medullary carcinoma tissue within an organ was associated with much higher levels of histaminase activity in that organ than would normally be present. For example, 690 units of histaminase/g was found in ovarian tissue containing foci of medullary carcinoma tissue compared to 13 units/g in normal ovary. In patients affected with both medullary carcinoma and pheochromocytoma, the medullary carcinoma tissue could be differentiated from the pheochromocytoma tissue by the high histaminase activity in the medullary carcinoma tissue and the low activity (< 20 units/g) in the pheochromocytoma tissue.

2. Rise in plasma histaminase activity in response to intravenous heparin administration. Plasma histaminase activity of man has been shown by other investigators to increase after parenteral administration of heparin. This response to heparin has been studied in detail with the sensitive radioassay for histaminase activity. At a dosage of 10 units/kg heparin i.v., at 10 minutes post injection plasma histaminase activity increased 1 to 2-fold; with 20 units/kg 2.5 to 6-fold; and with 75 units/kg 10 to 40-fold. Peak activity was reached on the average at 10 minutes following injection with all doses used. Clearance of the enzyme activity from plasma was exponential after all doses, and return to baseline levels was reached within two hours with the 20 unit/kg dose.

The above time course of the increase in plasma histaminase activity following heparin administration resembled that observed by other investigators for lipoprotein lipase. The appearance of histaminase activity in plasma was investigated in patients with Type I hyperlipoproteinemia and other hyperlipoproteinemias. At a dose of 10 units heparin/kg 4 patients with this disorder showed at 10 minutes plasma histaminase activities of 0, 0, 0.2, and 0.4 units/ml plasma. In contrast, the mean increase in histaminase activity in 21 normal individuals was 1.5 ± 0.5 units/ml (±2 SEM). With 75 units heparin/kg, 3 Type I patients showed an increase of 0.8, 4.4, and 4.7 units/ml plasma whereas the increase in histaminase activity in 10 normals ranged from 6.8 to 49.8 units/ml. In 2 patients with Type IV hyperlipoproteinemia and in 5 patients with Type V disease, histaminase activity increased from

0.9 to 6.0 units/ml after 10 units heparin/kg. One patient with Type IV disease failed to respond to 10 units heparin/kg and also had a low response (2.8 units/ml) to 75 kg. In vitro studies showed that concentrations of protamine or heparin that were sufficient to inhibit lipoprotein lipase had no effect on histaminase activity; 1 M sodium chloride, inhibited histaminase activity as well as lipoprotein lipase activity.

3. In vivo studies with aminoguanidine. A preliminary evaluation of the therapeutic effects of the histaminase inhibitor, aminoguanidine, in a patient with widely disseminated medullary thyroid carcinoma was briefly described in the previous report, NHLI-162(c). Regression of tumor was not achieved, but an increase in appetite, associated with a gain in weight, was observed. We have received recently a report from another center indicating that aminoguanidine had a beneficial effect on appetite in one patient with medullary carcinoma of the thyroid. Thus, we decided to evaluate the effect of aminoguanidine on appetite and growth in animals.

In two separate experiments (18 and 34 days duration) involving a total of 16 rats in each of the control and treatment groups, a statistically significant increase in food consumption was seen with a daily dose of 50 mg/kg aminoguanidine sulphate given perorally by stomach intubation. A 10 mg/kg dose also produced an average increase over control values in each study but failed to reach statistically significant levels. Weight gain was significantly greater in the 50 mg/kg group in the second experiment and all treatment groups showed an average weight increase greater than controls. In a third experiment, involving 15 animals each in a control group and a group receiving 50 mg/kg of aminoguanidine, food consumption and weight gain were slightly greater in the treatment group but the difference was not statistically significant. The concentration of aminoguanidine in tissues of chronically treated animals was highest in intestine and kidney and was present in lower amounts in salivary gland, liver and thymus. The drug was not detected in brain. Histaminase activity was inhibited by more than 90% in the intestine and thymus of aminoguanidine treated animals.

Significance: 1) The recent studies have confirmed that histaminase activity is an excellent biochemical marker for medullary thyroid carcinoma tissue. A relationship between detection of high histaminase activity and the presence of tumor cells has been further suggested. Use of measurements of histaminase activity in serum and tissues should continue to prove useful as a diagnostic and prognostic tool in this disease.

2) The apparent abnormality of plasma histaminase response to heparin in patients with Type I hyperlipoproteinemia and possibly in the patients with Type IV disease was a surprising finding. Such measurements may prove a useful adjunct to the measurements of lipoprotein lipase in detection of Type I disease.

3) The aminoguanidine studies in rats have in general paralleled the initial observations made in patients receiving aminoguanidine. The apparent direct effect of this drug on appetite might suggest utility for this drug in treatment

of anorectic patients with malignancies and other chronic disorders. There were no apparent side-effects from any dose of aminoguanidine given.

Proposed Course of Project: 1) Studies on histaminase activity in medullary carcinoma of the thyroid will be continued to examine further the exact nature and origin of the enzyme. 2) The reason for the deficiency of histaminase release in patients with Type I hyperlipoproteinemia will be investigated. The role, if any, of histaminase in lipolysis and the possible use of histaminase determinations in the diagnosis of lipid disorders will be explored. 3) Studies will be continued in animals to determine if there is a significant effect of aminoguanidine on appetite.

Honors and Awards: None

Publications:

1. Baylin, S.B., Beaven, M.A., Engelman, K., and Sjoerdsma, A.: Elevated histaminase activity in medullary carcinoma of the thyroid gland. New Eng. J. Med. 283: 1239-1244, 1970.

Serial No. NHLI-81(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on 6-Azuridine Triacetate (Azaribine) in Animals and Man.

Previous Serial Number: NHLI-160

Principal Investigator: Harry R. Keiser, M.D.

Other Investigators: Milan Slavik, M.D., Ph.D., Walter Lovenberg, Ph. D., Robert I. Henkin, M.D., Stephen Baylin, M.D., and Albert Sjoerdsma, M.D., Ph. D.

Cooperating Units: None

Project Description:

Objectives: 6-Azuridine triacetate is a potent pyrimidine antimetabolite, for oral use, developed in Czechoslovakia and in the United States. The drug inhibits de novo pyrimidine synthesis by blockade of the enzyme orotidine-5-phosphate decarboxylase. It has shown therapeutic efficacy in patients with psoriasis, mycosis fungoides, rheumatoid arthritis, chorioepithelioma, polycythemia vera and viral eye infections. An abnormal pattern of amino-aciduria was noted in patients and animals receiving the drug. We showed previously in animals that this was not a renal effect but that the drug produced alterations in serum amino-acids resembling three inborn metabolic diseases in man; ß-alaninemia, homocystinuria and cystathioninuria. Our animal studies had also indicated that Azaribine administration produced changes in zinc metabolism and in taste acuity. Thus, our objectives were to administer Azaribine to patients with scleroderma to study; 1) amino-acids in blood and urine to determine if similar biochemical alterations occurred in man as in animals; 2) possible beneficial effects of this drug in patients with scleroderma; 3) effects on taste acuity; and 4) effects on metabolism of copper and zinc. We also sought information from animal studies about the effects of Azaribine on the activity of enzymes essential in the metabolism of sulfhydryl-containing amino-acids.

Methods: Seven women, aged 44 to 62 years, with scleroderma were given Azaribine (Calbiochem) orally in equal doses each 8 hours for 7 day periods, beginning with 3 gm daily and increasing by 3 gm daily each 7 day period for a total of 4 periods or 28 days. Urine collections and blood samples were obtained during an initial control period and on the last 2 days of each period. Amino-acids were analyzed using standard techniques for analysis of

physiologic fluids on a Beckman 120C amino-acid analyzer. Analyses for copper and zinc were done by atomic absorption spectrophotometry according to the method of Meret and Henkin (Clin. Chem., In press). Measurements of taste acuity were obtained by use of techniques reported in detail previously by Dr. Henkin. Evaluation of the effects of Azaribine on scleroderma included measurements of range of joint motion, pulmonary function tests, and measurements of proline hydroxylase activity and the physico-chemical properties of collagen in punch biopsies of skin before and at the end of drug therapy. Eight rats were given either Azaribine, 1 gm/kg daily, or water by gastric gavage for 10 days and the activity of cystathionine synthetase in samples of their liver was determined according to the method of Mudd et al (J.B.C. 240.4382, 1965).

Major Findings: After one week of Azaribine administration to patients at a dose of 3 gm daily only minimal changes were found in serum amino-acids. After 2 weeks of therapy two amino-acids usually absent from human serum, homocystine and β-alanine appeared and significantly increased levels of threonine, glycine, methionine and histidine were found. The concentrations of these amino-acids in serum increased with the Azaribine dose reaching a pleateau usually at 9 gm daily. At this dose the level of homocystine was 41.4 ± 19.1 nM/ml of serum (mean ± SEM) and that of β-alanine was 23.7 ± 3.6. Analysis of urine revealed the expected spill over of these amino-acids into the urine. All of the patients had some anorexia and became slightly anemic at the high dose of Azaribine. Three of the patients had definite subjective improvement and mild objective improvement in their disease. Median detection thresholds for the four tastants prior to treatment were not different from normal, although median recognition thresholds for urea were slightly elevated. During drug therapy there was no change in median detection or recognition thresholds except for the recognition of sour, which was significantly increased. No subjective changes in taste acuity were reported by the patients. Azaribine administration to patients was associated with decreases in serum copper from 142 ± 6.9 µg/100 ml (mean ± SEM) to 121 ± 8.8 and in serum zinc from 79 ± 5.4 to 60 ± 3.9 µg/100 ml.

Azaribine administration to rats produced an inhibition of 42% in liver cystathionine synthetase activity when compared to controls. There was no effect of Azaribine on this enzyme in in vitro studies.

Significance: Azaribine administration to man produces significant alterations in the metabolism of a number of different amino-acids. The mechanism for these changes seems to be an inhibition of enzymes which are dependent on pyridoxal phosphate. The finding that the pyridoxal dependent enzyme cystathionine synthetase is inhibited by Azaribine in vivo but not in vitro indicates that it is not the drug per se but most probably a metabolite or an induced metabolic change which is responsible for the changes observed. Of major interest is the finding that Azaribine administration produces reversible changes in serum and urine amino-acids which resemble several inborn metabolic diseases. This may lead to increased understanding of these disorders.

There was some evidence of a beneficial effect from Azaribine on scleroderma. However, the study was not long enough to evaluate this aspect adequately.

The objective changes in taste acuity produced by Azaribine may be related to the changes noted in serum zinc and copper. However, there were no subjective changes in taste acuity observed in any of the patients and this should not be a problem in patients treated with this drug.

Proposed Course of Project: 1. Administration of Azaribine to patients with scleroderma for periods of 6 months or more to evaluate a possible beneficial effect.

2. Measurement of pyridoxal phosphate levels to determine if the enzyme inhibition produced by Azaribine administration is due to a deficiency of this necessary cofactor.

3. Further study of some of the specific amino-acid abnormalities noted in patients with scleroderma.

Publications: None

Serial No. NHLI-82(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolism of Hydroxyproline and Collagen

Previous Serial Number: NHLI-161(c)

Principal Investigator: Harry R. Keiser, M.D.

Other Investigators: I. Kelman Cohen, M.D.
Albert Sjoerdsma, M.D., Ph.D.
Charles Vogel, M.D.

Cooperating Units: Solid Tumor Centre, Uganda Cancer Institute, Kampala, Uganda; NCI

Project Description:

Objectives: 1. Development of methods for studying collagen metabolism in man and animals. 2. Application of these methods to the study of collagen metabolism in normal man, healing wounds, and the pathogenesis of various collagen diseases. 3. Study of the effect of various agents on wound healing and the selection and evaluation of agents for treatment of collagen diseases, especially scleroderma. 4. Elucidation of the biochemistry of keloids and hypertrophic scars, two examples of overabundant wound healing. 5. Determination of the origin of protocollagen proline hydroxylase (PPH) in the blood and its clinical significance.

Methods: The amino acid hydroxyproline (Hyp) is a unique marker for collagen. Methods for assaying Hyp in urine, plasma and tissue have been developed and tested in this laboratory and described previously. Methods were also developed for studying the physicochemical properties of collagen in tissues, for measurement of the rate of collagen synthesis in tissues, and for biochemical studies of standardized healing wounds. These techniques have been applied to studies of wound healing in normal man and laboratory animals, to studies of wound healing in patients with scleroderma, and to studies of abnormal wound healing, i.e. keloid and hypertrophic scar.

Recently we have set up the technique for measuring collagenase activity in tissue samples according to methods developed in NIDR. Tissue samples are grown for 5 days in tissue culture with daily changes of media. The harvested media is pooled and its collagenolytic activity measured by incubating it with a radioactively labelled collagen gel and then measuring the radioactivity released. This method coupled with our techniques for measuring collagen synthesis allows us to evaluate both collagen formation and breakdown in tissue.

We have devised a simple technique for administering controlled concentrations of volatile anesthetic agents to 4 rabbits at once. This is accomplished by utilizing a Foregger anesthesia machine to vaporize the anesthetic, mix it with oxygen or room air and to control all flow rates. The gas mixture is delivered into a large metal animal cage totally enclosed in a large polyethylene bag. Gas is exhausted from the cage by a one way valve made from a piece of Penrose-drain.

Major Findings: Our studies of serum PPH-activity have now been extended to over 450 patients from both the U.S.A. and Africa. Our normal level is up to 570 dpm/ml/hr. Very high levels (from 1000 to 14,362) were found in 56% and elevated levels (> 570) were found in 70% of 54 patients with hepatoma. As a diagnostic test PPH level ranks with alpha-feto-protein, another serum factor found in hepatoma, which was positive in 59% of these patients. Elevated levels of serum PPH were found also in 34% of 32 patients with neoplasms metastasizing to liver, in 31% of 35 patients with hepatitis and in 10% of 49 patients with cirrhosis. Serum PPH levels were elevated in 85% of 13 newborns and in all of 10 patients after surgery under anesthesia with the fluorinated hydrocarbons, methoxyflurane or halothane. Serum PPH activity was not increased in patients with any of the common "connective tissue" diseases, in patients with tumors metastasizing to bone or with primary bone tumors, Paget's disease or after anesthesia with nitrous oxide. There was a general correlation between serum PPH levels and serum alkaline phosphatase levels when elevations of the latter were of liver origin but not when they were from bone.

We have found that rabbits show similar changes in serum PPH and other enzymes after methoxyflurane exposure, as does man. This occurs in rabbits at inspired gas concentrations which are so low that the animals are only sleepy and not really anesthetized. Serum PPH-activity rose rapidly in animals exposed to 0.3% methoxyflurane for 2 hours from control levels of 916 $\pm$ 366 dpm/ml/hr (mean $\pm$ SEM) to peak levels of 7612 $\pm$ 1164 ($p < .01$) at 12 to 28 hours after the start of the experiment. It returned to initial levels within 48 to 96 hours. Serum levels of serum glutamic oxaloacetic transaminase (SGOT) rose more slowly from control levels of 40 $\pm$ 4 S.F. units to peaks at 48 to 96 hours of 402 $\pm$ 124 ($p < .02$). A similar change was noted in levels of serum glutamic pyruvate transaminase (SGPT) but there was no change in serum levels of alkaline phosphatase. Rabbits exposed to N_2O for 2 hours showed no significant changes in serum PPH activity. When cycloheximide (Calbiochem), a potent inhibitor of protein synthesis, was given intraperitoneally to rabbits in doses of 1 mg/kg B.W., it alone produced significant elevations in serum activities of PPH, SGOT, and SGPT. When rabbits received both cycloheximide and methoxyflurane the effects on serum levels of PPH, SGOT, and SGPT were additive. Assay of the livers of these animals for PPH-activity showed no significant differences between any of the 4 groups, i.e., controls, cycloheximide alone, methoxyflurane alone and cycloheximide plus methoxyflurane.

PPH-activity in tissues is a good indicator of the rate of collagen synthesis. We have expanded our data on PPH-activity in punch biopsies of skin to include 10 normals, 20 patients with scleroderma, one patient with morphea

(localized plaques of scleroderma) and 17 patients with miscellaneous, non-connective tissue diseases. PPH-activity is significantly higher in apparently uninvolved parasacral skin from patients with scleroderma when compared to that of normals, being 364 ± 24 vs 180 ± 16 dpm/mg dry wt/hr (mean ± SEM). PPH-activity was even greater, 626 ± 60 in samples of obviously involved forearm skin of patients with scleroderma. The average levels of PPH-activity in both forearm and parasacral skin of patients with miscellaneous diseases were not significantly different from that of controls but were significantly different from that of patients with scleroderma. PPH-activity in skin biopsies from a patient with morphea (localized scleroderma) was 216 dpm/mg/hr in uninvolved skin, 518 in the center of a plaque of morphea, and 848 in the advancing edge of that plaque.

The mean level of PPH-activity in keloids from 8 patients, 4015 ± 626 dpm/mg/hr (mean ± SEM), is greater ($.02 < p < .05$) than that in hypertrophic scars from 6 patients, 1713 ± 511. The mean levels of PPH-activity in both are greater ($p < .01$) than those in skin from 10 normal subjects, 179 ± 16, and in normal appearing scars from 5 patients, 209 ± 35. There is no significant difference in PPH-activity between skin of normal subjects and normal appearing scars. However, mean PPH-activity adjacent to either keloid or hypertrophic scar in 10 patients, 574 ± 112, is significantly less than that of both keloid and hypertrophic scar ($p < .01$) yet significantly higher than that of normal scar and skin of normals ($p < .01$). While only very preliminary results are available, collagenase activity in keloids and hypertrophic scars appears to be considerably elevated over that in normal skin.

Significance: All evidence indicates that elevated PPH-activity in serum is of hepatic origin. We believe that serum PPH-activity constitutes a new and sensitive indicator of liver cell injury. We have shown that the rabbit serves as a good animal model for studying effects of anesthetic agents. The use of serum PPH levels adds a new dimension for analyzing the hepatotoxic effects of these agents. The fantastically high levels of PPH-activity in serum of patients with hepatoma makes it a useful tool in the diagnosis of this neoplasm. While relatively rare in the U.S., hepatoma is one of the most common malignant tumors in other countries. Serum PPH levels may prove useful in following either chemotherapy or surgical extirpation of this rapidly fatal malignancy.

The finding of elevated levels of PPH-activity in the skin of patients with scleroderma and morphea confirms the clinical impression of increased collagen synthesis. This is an important finding since it occurs in both obviously involved and apparently uninvolved sclerodermatous skin. Whether this change is primary or secondary in the pathogenesis of this disease remains to be elucidated.

The increased PPH-activity in keloids and hypertrophic scars indicates that the rate of collagen synthesis is markedly elevated in these states. This helps explain the excessive collagen present in these examples of overabundant wound healing. It also provides a yardstick for evaluating drug interventions aimed at controlling this disfiguring complication.

Proposed Course of Project: 1. Continued studies of serum PPH-activity in patients, especially as a tool for evaluating therapy in patients with hepatoma.

2. Studies of other anesthetic agents for their effects on serum PPH levels in man and on rabbits. Special attention will be given the fluorinated hydrocarbons which have shown sporadic liver toxicity in man.

3. Further characterization of the biochemistry of keloids and hypertrophic scars with studies of the effects of various therapeutic interventions upon these states.

Honors and Awards: None

Publications:

1. Stein, H.D. and Keiser, H.R.: Collagen metabolism in granulating wounds. J. Surg. Res. In press.

2. Keiser, H.R., Stein, H.D., and Sjoerdsma, A.: Increased protocollagen proline hydroxylase activity in sclerodermatous skin. AMA Arch. Dermatol. In press.

Serial No. NHLI-83
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Pharmacological Agents on Central Neurotransmission Processes using the Spinal Cord as a Simple Model System.

Previous Serial Number: None

Principal Investigator: B. V. Clineschmidt, Ph. D.

Other Investigators: Albert Sjoerdsma, M.D., Ph. D.

Project Description::

Objectives: Anatomical, biochemical and pharmacological studies support the view that serotonin (5-HT) and norepinephrine (NE) serve as neurotransmitter substances in the spinal cord and brain. Because of its relative anatomical simplicity and the fact that spinal reflexes are stable over long periods of time which are often required for full development of a drug's action, the spinal cord would seem to provide a good model for investigating the effects of substances believed to influence central monoamine neurotransmission. The specific objective of the study now in progress was to determine if tricyclic antidepressants affect the action of 5-HT on spinal reflexes.

Methods: Spinal reflex activities from an L_7 ventral root are amplified and displayed on an oscilloscope. The reflex is evoked by stimulating the corresponding dorsal root in unanesthetized spinal cats. The 5-HT precursor 5-hydroxytryptophan (5-HTP) is injected to elevate the level of 5-HT in the cord. Imipramine or a related agent is administered either before or after 5-HTP.

Major Findings: 5-HTP (50 mg/kg) produces a slowly developing increase in the height of the spinal monosynaptic reflex (MSR). Sixty minutes after 5-HTP, the height of the MSR is 250% of the pre-drug size. The tricyclic antidepressant imipramine (5 mg/kg) causes a decrease of about 25% in the height of the MSR. Following pretreatment with imipramine, 5-HTP increases the height of the MSR to nearly 750% of its control size within sixty minutes after injection. In contrast to its effect in untreated animals, imipramine given sixty minutes after 5-HTP causes a very rapid increase of more than four-fold in the height of the MSR. In cats with chronic spinal transection (T_8-T_9), 5-HTP produces its usual increase in the monosynaptic spike height. However, imipramine administered after 5-HTP causes a decrease instead of an increase in the height of the MSR.

The enhancement of the MSR produced by 5-HTP plus a tricyclic agent is antagonized by the 5-HT antagonists cinanserin (2.5 mg/kg) and methysergide (1-2 mg/kg). The interaction between 5-HTP and imipramine does not occur in animals pretreated with RO 4-4602 to prevent decarboxylation of 5-HTP to 5-HT. Other antidepressants such as amitriptyline and desmethylimipramine have qualitatively the same effects on the MSR as imipramine both in untreated and 5-HTP pretreated animals.

Significance: The observations indicate that tricyclic antidepressants of the imipramine type enhance a central effect of 5-HT, i.e. the increase in the MSR following 5-HTP administration. A similar effect on the actions of 5-HT at higher levels in the C.N.S. might be responsible for the therapeutic and some of the side effects of tricyclic antidepressants. Although much of the current thinking on the mechanism of the antidepressant action of these compounds involves central noradrenergic processes, our results show that central actions of 5-HT must also be considered.

5-HT has been shown to have a beneficial effect on hypotonia in Down's syndrome. Its effectiveness here might depend upon influencing spinal reflex activity in which case the combination of 5-HTP and a tricyclic agent could prove superior to giving 5-HTP by itself.

Proposed Course of Project: 1) 5-HT levels. To this point, we have assumed that 5-HTP administration increases spinal cord 5-HT. 5-HT levels in lumbosacral cord will be assayed before and after 5-HTP. 2) Mechanism of interaction. Tricyclic antidepressants inhibit uptake of 5-HT into monoamine nerve terminals. We plan to determine if the order of potency of tricyclic compounds in inhibiting 5-HT uptake corresponds to their order of potency in enhancing the 5-HTP-induced increase in the spinal MSR. 3) Catecholamine precursors. The NE and dopamine precursor L-dihydroxyphenylalanine (DOPA) affects spinal reflexes. The combination of DOPA with tricyclic agents will be studied. Also, the effect of imipramine on the spinal actions of dihydroxyphenylserine (precursor of NE only) and alpha-methyldopa may be investigated.

Publications: None

Serial No. NHLI-84(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Clinical Investigation of Cardiovascular Drugs

Previous Serial Number: NHLI-158(c)

Principal Investigator: David Horwitz, M.D.

Other Investigators: Albert Sjoerdsma, M.D., Ph.D., Griff T. Ross, M.D., Ph.D., B. Van Clineschmidt, Ph.D., Harry S. Margolius, M.D., Ph.D., and Richard Wyatt, M.D.

Cooperating Units: Endocrinology Service Unit, Reproduction Research Branch, National Institute of Child Health and Human Development; Adult Psychiatry Branch, National Institute of Mental Health.

Project Description:

A. Influence of Various Central Nervous System Drugs on Plasma Gonadotropin Levels

Objectives: In animals it has been possible to demonstrate the influence of the central nervous system on gonadal function and to modify ovulation by the use of drugs which alter the levels of amine neuromediators in the central nervous system. It is not known, however, whether similar effects can be obtained in man or which amine mediator plays a predominant role. Our studies have been concerned with the influence of drugs which alter central nervous system amines on the blood levels of pituitary gonadotropins as measured by radioimmunoassay. Initial observations were made in postmenopausal women or in males because they have relatively stable levels of gonadotropins. Such studies, utilizing the drugs parachlorophenylalanine, reserpine, pargyline, desipramine, L-dopa, methyldopa, α-methyl-5-hydroxytryptophan, α-methyl-para-tyrosine and α-methylphenylalanine, were inconclusive.

We have since attempted observations in cycling premenopausal women. Because the critical peaks of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) which accompany ovulation are present only during one to two days per cycle, it has been necessary to obtain daily samples over many months.

Major Findings: Results are available from three women, each followed through six consecutive months during which methyldopa therapy was alternated with a control drug. In each instance there was pronounced reduction of cyclic peaks of LH during therapy with methyldopa and lesser effects on FSH levels.

Significance: This is the first demonstration that gonadotropin levels can be altered in man by drugs which are not hormones. It is an initial step toward the goals of defining the role of the central nervous system in the control of gonadotropins in man and of developing new methods for altering fertility.

Proposed Course of Project: Studies will be pursued in additional subjects and with other agents. Simultaneous measurements of plasma estrogens and progesterone will be made to determine how feedback controls have been altered.

B. Studies in Vascular Headache

Objectives: A role of serotonin in migraine headaches has been suggested by reports of changes in plasma levels of serotonin and the urinary excretion of its degradation product, 5-HIAA, during attacks of migraine; the clinical usefulness of the serotonin antagonists cyproheptadine and methysergide makes the relationship more plausible. We therefore undertook short-term observations of the effects of an inhibitor of serotonin synthesis, parachlorophenylalanine (PCPA), in hospitalized patients with migraine; the drug was well-tolerated and appeared to be beneficial, but results were inconclusive. As a consequence a controlled long-term outpatient study of the effects of PCPA in migraine was begun.

Major Findings: Eight patients with frequent episodes of typical migraine, which responded poorly to conventional medications, have so far been entered into a 6 month double-blind, cross-over study comparing the effects of PCPA with those of a placebo. Analysis of therapeutic effects is not yet available. Two of the eight, each with a history of allergy, developed pronounced drug-related reversible eosinophilia and were withdrawn from the study without adverse effects.

Proposed Course of Project: The study will be pursued in additional subjects.

C. Role of Brain Amines in Sleep

Objectives: Previous studies in man have shown that the rapid eye movement (REM) phase of sleep is decreased when serotonin synthesis is reduced by the drug, parachlorophenylalanine (PCPA). REM sleep is restored in such subjects when the block in serotonin synthesis is overcome by giving the serotonin precursor, 5-hydroxytryptophan. Similar results have been obtained in animals with a variety of pharmacologic manipulations which alter serotonin levels. In animals it has been possible to demonstrate monophasic sharp increases in the electrical activity of the pontine oculomotor nuclei, lateral geniculate nuclei and visual cortices beginning shortly before and persisting through the REM phase of sleep; this electrical activity designated as pontine-geniculate-occipital (PGO) spikes, has been hypothesized to be critically necessary for REM sleep and is grossly disturbed by treatment of animals with PCPA. A possible

analogue to PGO spikes designated as phasic integrated potentials (PIP) is under study in patients during normal sleep and during treatment with PCPA.

Major Findings: Studies have been completed in two subjects with migraine who received PCPA as possible therapy. During treatment with PCPA there was a decrease in PIP counts in REM sleep and an increase during non-REM sleep, yielding a four-fold rise in the non-REM to REM ratio of PIP activity. Such changes resemble those of PGO spikes in animals receiving PCPA, supporting the hypothesis that PIP waves in humans are analogous to PGO waves in cats.

Proposed Course of Project: Studies are being pursued in additional subjects.

D. Reactivity of Human Temporal Arteries

Objectives: Increased vascular reactivity has been reported in hypertensive humans by studies utilizing a variety of techniques. Interpretation has been difficult because of differing levels of initial tone or reflex feedback and differing wall-lumen ratios in hypertensive and normotensive subjects. In an attempt to investigate this problem in a simplified system, we initiated a study of the *in vitro* responses of helical strips prepared from biopsied human temporal arteries. This technique had previously been used in this department to study aortic strips from genetic hypertensive and normotensive rats; there were no systematic differences in responses to the sympathetic neuromediator norepinephrine nor in the dissociation characteristics of alpha receptors in the two groups.

Major Findings: Studies of temporal arteries from seven normotensive neurosurgical patients have been completed. Optimal tension varied from 1200 to 5000 mg and was achieved when strips were stretched 35 to 45% above initial lengths. Average concentrations eliciting half-maximum responses were 3.3×10^{-5} mg/ml for norepinephrine and 2.6×10^{-4} mg/ml for phenylephrine. The negative log of the molar concentration of phentolamine which reduced the effect of a double dose of phenylephrine to that of a single dose (pA_2) was 7.8; this is comparable to values obtained in studies on alpha adrenergic receptors in blood vessels of animals.

Significance: Development of this technique promises to permit resolution of the question of hypersensitivity of vascular smooth muscle in hypertensive humans and will permit comparison of receptors in normotensive and hypertensive subjects.

Proposed Course of Project: The observations on normotensives will be expanded and comparison made with vessels from hypertensive subjects.

E. Cardiovascular Effects of L-dopa

Objectives: Blood pressure reduction has been widely reported as a side-effect when patients with Parkinson's disease are treated with large doses of L-dopa.

Observations in animals have suggested that L-dopa and its analogue, the antihypertensive agent methyldopa, exert their hypotensive effects via the central nervous system through conversion to amines. The compound, L-α-methyl-α-hydrazino-3,4-dihydroxyphenylpropionic acid (MK-486), blocks the decarboxylation of L-dopa and methyldopa to amine products in peripheral tissues but does not enter the central nervous system. In so doing it has been reported to promote the accumulation of the L-dopa-derived amine, dopamine, in the brain and to reduce the dose requirements for treating Parkinson's disease. Use of MK-486 thus offers an opportunity to distinguish between central and peripheral loci for the hypotensive effects of L-dopa and methyldopa in patients.

Major Findings: Four patients with mild hypertension were studied while receiving L-dopa alone in daily doses of 2.0 gm and also after the addition of MK-486 in a daily dose of 150 mg (one subject) or 500 mg (3 subjects). Two showed low grade blood pressure reduction while receiving L-dopa alone; there was no evidence of enhancement of hypotensive effects after the addition of MK-486.

Proposed Course of Project: Further studies are planned using higher doses of L-dopa and MK-486 and combining MK-486 with methyldopa.

Honors and Awards: None

Publications: None

Serial No. NHLI-85(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Biological Role of Histamine: Examination of Histaminase Activity in Rat Tissues and Its Release by Heparin

Previous Serial Number: NHLI-153

Principal Investigator: Michael A. Beaven, Ph.D.

Other Investigators: Stephen B. Baylin, M.D.
Wybren De Jong, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: As a part of the study of the metabolism of histamine in the body, the enzyme histaminase was examined in detail. Histaminase is thought to be identical to the enzyme diamine oxidase by some workers while the enzymes are thought to be separate entities by other workers. The objectives of this work were (1) to determine whether histaminase, as measured by the release of tritium from β-^{3}H-histamine (see Project Report NHLI-153, 1969-70), was the same enzyme as that which deaminates putrescine (diamine oxidase) and whether the assays of the two enzyme activities were interchangeable; (2) to establish the distribution of histaminase in tissues; and (3) to study the release of histaminase from tissues by heparin. Heparin is known to raise histaminase levels in plasma of experimental animals and man; the histaminase is thought to be released from liver in guinea pig and from intestine in rat. The present studies in rat examined the release of histaminase by heparin in a number of tissues and the kinetics of this release. These studies were intended to complement the investigation of the release of histaminase by heparin in man.

Methods: Histaminase activity was determined by measurement of the release of tritium from β-^{3}H-histamine as described in Project Report NHLI-153, 1969-70. Diamine oxidase activity was determined by a modification of the method of Okujama and Kobayashi (Arch. Biochem. Biophys. 95: 242, 1961) with β-^{3}H-putrescine as substrate.

Major Findings: 1) Biochemical studies of histaminase. The data showed that histaminase activity was associated with diamine oxidase activity in all the tissues studied. The deamination of β-^{3}H-histamine and β-^{3}H-putrescine was inhibited to an equal extent by aminoguanidine (I_{50} 2.5 x 10-8 M). The deamination of β-^{3}H-histamine was inhibited by putrescine and the deamination

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of β-^{3}H-putrescine was inhibited by histamine. Kinetic studies showed that the inhibition was competitive. The studies also indicated that the assay of histaminase activity was interchangeable with the assay of diamine oxidase activity although the histaminase assay was more sensitive and reproducible.

There were dissimilarities in the two enzyme activities. The effects of pH and substrate concentrations on the deamination of β-^{3}H-histamine and β-^{3}H-putrescine were different. Also, tritium was released from β-^{3}H-histamine but not from β-^{3}H-putrescine. The possibility that β-^{3}H-histamine and β-^{3}H-putrescine were deaminated by the same enzyme but by different mechanisms was reviewed in Project Report NHLI-153, 1969-70.

Other studies showed that histaminase could be differentiated from plasma amine oxidase (benzylamine oxidase) in human plasma and from purified beef plasma amine oxidase by β-^{3}H-histamine since this amine was not a substrate for either plasma amine oxidase.

2) Distribution of histaminase activity in tissues. Levels of histaminase activity in rat tissues ranged from 0 to 8000 μμmoles histamine deaminated/g tissue/hr as follows:

Rat Tissue	Histaminase Activity*
G.I. tract; appendix	8000
small intestine	3300-6200+
stomach (glandular)	250
Thymus	500-1100+
Adrenals	150-350
Pancreas	41
Lung	38
Bone marrow	28
Spleen	27
Liver, kidney, prostate	7
Heart	5
Plasma	2.8-4.6+
Submaxillary gland, skin	
testicles, brain	0

*μμmoles histamine deaminated/g tissue or ml plasma/hr, mean value from several experiments.
+These values indicate the range of values from different experiments.

The high levels of histaminase activity in intestinal tract, thymus and adrenals were found in both normal and germ-free rats. The histaminase levels in thymus diminished with age and were decreased by treatment with dexamethasone (see below). Interesting differences in histaminase activity were observed in thymus of normotensive Wistar and spontaneously hypertensive (SH) Wistar rats as seen below:

Rat	Histaminase Activity in Thymus*			
Age:	6 wk	8 wk	10 wk	20 wk
Wistar, normal	497±26(5)	456±38(11)	438 (2)	307±49(6)
Wistar, SH	1260±89(8)	1100±54(16)	1077±99(5)	299±32(6)
Sprague-Dawley, control		960±160(5)		
Sprague-Dawley, with dexamethasone+		263±45(5)		

*μμmoles histamine deaminated/g tissue/hr, mean value ± S.E. (number of animals).

+These animals were treated with dexamethasone, 500 μg/kg, for 3 days.

In guinea pigs, high levels of histaminase activity were found in lymph nodes, spleen and thymus. Histaminase activity was also found in lymphocytes isolated from spleen and bone marrow but not in circulating lymphocytes of guinea pigs.

3) Release of tissue histaminase by heparin. Heparin reduced histaminase activity in rat intestine and adrenals but not in thymus. The extent of the depletion was dependent upon the dose of heparin. Doses of 4,000, 20,000 and 40,000 units/kg, i.v., reduced histaminase activity in intestine by 40%, 63% and 76%, respectively, with maximum depletion 15 to 30 min after the injection of heparin. Maximum levels of histaminase activity in plasma, 200, 585 and 538 μμmoles histamine deaminated/ml/hr, were seen 15 to 30 min after injection of 4,000, 20,000 and 40,000 units heparin/kg. Histaminase activity in plasma declined to near normal, 2-4 μμmoles/ml/hr, by 5 hr. At this time, the enzyme activity in the intestine began to rise and by 10 hr the activity was back to normal, 4,200 μμmole/g/hr. The data showed that a) histaminase in intestine was almost completely depleted by heparin; b) the enzyme in intestine was synthesized and was turned over at a rapid rate; c) histaminase was removed from plasma within 5 hr; and d) histaminase in thymus was impervious to the depleting action of heparin, which suggested that the enzyme in thymus may be different from that in intestine.

Significance: The studies have shown that histaminase and diamine oxidase are similar or identical enzymes in various tissues and that the assays of these two enzyme activities are interchangeable. This finding has enabled us to correlate the data obtained with the assay of histaminase activity, which is the more sensitive and reproducible assay, with the previously published findings with the assay of diamine oxidase activity. The distribution of histaminase activity in rat tissues indicated that high histaminase activity was present in thymus, which has not been previously described as a source of histaminase, as well as in adrenals and in intestine. The studies of the heparin release of histaminase have confirmed that in rat the intestine is the primary source of histaminase; the data have shown that the intestine

can be almost completely depleted of histaminase and that the enzyme is turned over at a rapid rate. A rapid turnover of histaminase has also been noted in medullary carcinoma of the thyroid tissue. The experiments with heparin in animals have provided necessary information for the interpretation of studies of heparin release of histaminase in man.

Proposed Course of Project: The significance of histaminase in thymus and lymphatic tissue will be examined, for example, by determining the factors which influence the levels of histaminase in these tissues. The animal studies with heparin have raised a number of important questions concerning the studies of histaminase release by heparin in man. One aspect that will be examined is whether the defect in release of histaminase by heparin in Type I hyperlipoproteinemia is due to an absence of enzyme in the intestine or due to a defect in release of the enzyme from intestine. The mechanism of the heparin release of histaminase will be studied in animals to provide a basis for the further study of the action of heparin on histaminase in man.

Honors and Awards: None

Publications:

1. Beaven, M.A. and Jacobsen, S.: A new assay for histaminase activity: Measurement of tritiated water from β(side chain label)-H^3-histamine. J. Pharmacol. Exptl. Therap. 176: 52-64, 1971.

Serial No. NHLI-86(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Catecholamines in Human Disease.

Previous Serial Number: NHLI-163(c)

Principal Investigator: Harry R. Keiser, M.D.

Other Investigators: Milan Slavik, M.D., Ph.D., Stephen Baylin, M.D., David Horwitz, M.D., and Albert Sjoerdsma, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: During the past year our research in the area of catecholamine metabolism has centered on evaluation and study of the clinical pharmacology of a new antihypertensive agent, MK-266. This drug is the chlorobenzyl ether of metaraminol. Upon metabolism, metaraminol is released and should function as a false neurotransmitter in sympathetic nerves and thereby lower blood pressure.

Methods: Catecholamines in blood and urine and catecholamine metabolites in urine were measured by methods developed and used in this laboratory for years. The determination of metaraminol in urine of patients receiving MK-266 was performed by a modification of the method of Pugsley and Johnson (J. Pharm. Pharmacol., 1968) as we have reported previously.

Major Findings: MK-266 has now been administered to 7 patients with essential hypertension. In the first 3 patients single oral doses as large as 400 mg had no effect on blood pressure. Six of the patients have received doses of 300 to 800 mg/day for periods of 4 to 8 days. Three of these patients had slight to moderate decreases in blood pressure, most marked usually in the standing position. Three patients developed significant changes in SGOT and SGPT levels during drug administration and one of these patients had concomitant nausea and emesis. These symptoms disappeared and abnormal laboratory tests returned to normal when the drug was stopped. One other patient had a maculopapular skin eruption on the second day of MK-266 administration. This disappeared within hours when the drug was stopped and did not return when the drug was restarted. No central nervous system symptoms were observed in any patients. We have been able to recover only about 1% of the administered drug as metaraminol in the urine of 5 patients tested.

Significance: MK-266 is an ether analog of metaraminol which is released slowly on metabolism. Metaraminol has no direct pressor activity of its own but is capable of releasing norepinephrine. Previous studies have indicated that continuous intravenous infusions of metaraminol lead to a lowering of blood pressure. MK-266 holds promise of permitting the oral administration of metaraminol. An antihypertensive effect would provide evidence in favor of the "false neurotransmitter concept". We have seen some blood pressure effect from MK-266 but because of unexpected liver toxicity we have not been able to administer large doses of drug. Larger doses of drug are desirable since only 1% can be recovered as the presumably active metabolite metaraminol. Thus, the clinical usefulness of the drug is still in doubt, and the validity of the false neurotransmitter concept is unaffected by these studies.

Proposed Course of Project: MK-266 will be administered carefully to a few more hypertensive patients to allow more complete elucidation of its potential usefulness.

2. A careful search will be made for other metabolites of this drug in urine of patients.

Honors and Awards: None

Publications: None

Serial No. NHLI-87(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Biological Role of Histamine: Metabolism of β-^{3}H-histamine in Man.

Previous Serial No.: None

Principal Investigator: Michael A. Beaven, Ph. D. and Stephen B. Baylin, M.D.

Other Investigators: Harry R. Keiser, M.D. and Albert Sjoerdsma, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: As part of the study on the role of histamine, the metabolism of histamine in man was investigated. This work was undertaken to determine the relative importance of the various enzymes known to metabolize histamine. With this information it is hoped that more effective agents can be chosen to block the metabolism of histamine in man. A major deficiency in our knowledge is whether histaminase (diamine oxidase) or monoamine oxidase is the more important enzyme in the metabolism of histamine. The present studies utilize the ability to distinguish between histaminase and monoamine oxidase activities by 1) the release of tritiated water from β-^{3}H-histamine by histaminase and 2) the formation of tritiated acidic metabolites of β-^{3}H-histamine by monoamine oxidase.

Methods: β-H-Histamine was prepared by decarboxylation of β-^{3}H-L-histidine with bacterial histidine decarboxylase. The preparation was purified in the laboratory and solutions for injection were prepared by the Pharmacy Department. The β-^{3}H-histamine was administered i.v. and urine was collected over 6-hour periods for the first 24 hours and for 24-hour periods on the 2nd and 3rd days after injection of the labeled amine. Aliquots were removed from each collection and frozen. Urinary metabolites of β-^{3}H-histamine were determined by the following procedures: 1) by ion exchange chromatography to separate acidic and basic metabolites; 2) by addition of unlabeled metabolites or histamine as carriers and the crystallization of various derivatives of these metabolites; 3) by solvent extraction techniques; 4) by thin layer and paper chromatography; and 5) by total tritium and volatile tritium (tritiated water) determinations. Various samples of authentic histamine metabolites were obtained from commercial sources and from Dr. H. Tabor and Dr. H. Bauer, NIAMD.

Major Findings: The data from six patients showed the following: 1) Seventy to eighty percent of the injected tritium label was excreted into urine during the first 6 hours; 95% or more of the tritium was eliminated within 24 hours after the injection of the labeled amine. Over a 3-day period all of the tritium could be accounted for as tritiated metabolites or tritiated water. There was no evidence of significant retention of β-^{3}H-histamine or labeled metabolites in the body. 2) The excretion of tritiated water accounted for 5 to 8% of the injected label. This indicated that the β-^{3}H-histamine was metabolized by histaminase to only a minor extent. 3) Small amounts of unchanged β-^{3}H-histamine, less than 3% of the injected tritiated amine, were present in the first 6 hour urine collection. 4) The major tritiated metabolites in urine were methylimidazole acetic acid (56 to 77% of the injected β-^{3}H-histamine) and imidazole acetic acid riboside (13 to 39%). These acidic metabolites accounted for greater than 70% of the tritium label. The fact that tritium was retained by these acidic metabolites indicated that monoamine oxidase was of major importance in the metabolism of β-^{3}H-histamine. 5) Administration of a monoamine oxidase inhibitor, pargyline (1 mg/kg), to one of the patients resulted in a small decrease (20%) in the amount of tritiated methylimidazole acetic acid excreted. Preliminary results indicated that administration of the histaminase inhibitor, aminoguanidine (10 mg/kg), resulted in the reduction of tritiated water formation and the disappearance of ^{3}H-imidazole acetic acid riboside in urine.

Significance: These studies have revealed that histaminase (diamine oxidase) plays only a minor role in the metabolism of histamine in vivo. The results suggest that the enzyme which methylates histamine to methylhistamine, histamine-N-methyl-transferase, and monoamine oxidase are the more important enzymes in histamine metabolism. This information provides a rational basis for the inhibition of histamine metabolism in man.

Proposed Course of Project: The studies will be continued to determine whether inhibitors of the enzymes histamine-N-methyltransferase and monoamine oxidase will block the metabolism of histamine and thereby increase tissue histamine. Since histamine is a vasodilator, it is hoped that such inhibitors will be of use in the treatment of vascular disorders such as Raynaud's disease. The use of these inhibitors will also be employed to determine whether histamine plays a normal role in vasodilation.

Publications: None

Serial No. NHLI-88(c)
1. Experimental Therapeutics Branch
2. Section on Experimental Medicine
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies of the Biological Role of Histamine: Application of the Enzymatic Assay of Histamine to the Measurement of Histamine in Urine

Previous Serial Number: NHLI-144

Principal Investigators: Michael A. Beaven, Ph.D.
Harry R. Keiser, M.D.

Other Investigators: Zdenka Horáková, Ph.D.
Stephen B. Baylin, M.D.
Albert Sjoerdsma, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: In order to further assess the role of histamine in man, the enzymatic assay of histamine (see Project Report NHLI-144, 1969-70) was modified for the direct measurement of histamine in urine. The study was undertaken to provide a simple method of detecting changes in the excretion of histamine. Previously published assays for histamine in urine are time-consuming and require some prior purification of the histamine. The enzymatic assay is specific and sensitive and can be performed on as many as 60 samples of untreated urine at a time. In the present study histamine excretion was determined in urine of control subjects and of a patient with mastocytosis, a condition associated with the release of large quantities of histamine. In addition, the effect of oral administration of L-histidine on histamine secretion in urine was studied.

Methods: The assay procedure, which is based upon the formation of ^{14}C-methylhistamine from histamine in the presence of S-adenosylmethionine-^{14}C-methyl and histamine-N-methyltransferase, is described in detail in Project Report NHLI-144, 1969-70. In the present studies, samples were removed from 24 hr urine collections and frozen until time of assay; 20 µl aliquots were taken for assay.

Major Findings: 1) Adaptation of assay to measurement of histamine in urine. Studies showed that a 20 µl aliquot of urine was an optimum quantity for the assay; larger volumes, 50 to 100 µl, resulted in a diminution, by 30 or 80% respectively, in the recovery of histamine as ^{14}C-methylhistamine. Unlabeled histamine added to the 20 µl urine sample was recovered quantitatively and reproducibly. The limit of sensitivity of the procedure was 0.1 ng histamine.

The levels of histamine in normal urine were lower than those reported previously in the literature when the fluorometric assay of histamine had been used. Possible reasons for the lower histamine levels found in the present work are: (1) the enzymatic assay is specific for histamine, whereas the fluorometric assay is subject to interference from compounds such as spermidine; (2) the isolation of histamine from urine is not required for the enzymatic assay and the possibility of histamine arising from decarboxylation of L-histidine is reduced. The daily excretion of L-histidine in urine is 160-260 mg/day; the spontaneous decarboxylation of 0.01% of the histidine could yield 10 to 20 µg histamine.

2) Histamine in normal urine. In 16 normal males and females, the levels of histamine ranges from 10 to 40 ng/ml urine or 3 to 40 µg/24 hr, mean value 11 µg/24 hr. An additional female had a value of 92 µg/24 hr.

3) Histamine in urine of a patient with mastocytosis. As a confirmation of the assay, histamine was measured in a patient with mastocytosis. Histamine levels ranged from 160-220 µg/24 hr. Histamine output in urine was increased to 310 µg/24 hr by oral administration of L-histidine, 4 x 10 g daily for 3 days. Urinary histamine excretion returned to pretreatment levels immediately upon cessation of L-histidine administration. Examination of histamine levels in two suspected cases of mastocytosis failed to show abnormality in histamine excretion.

4) Effects of oral administration of L-histidine on urinary histamine levels. A further confirmation of the assay was that the administration of L-histidine produced a pronounced rise in urinary histamine levels. In a normal volunteer, histamine levels rose from 28 µg to 297 µg/24 hr, and in one female patient with Raynaud's disease, a rise from 19 µg to 180 µg/24 hr was observed after administration of 40 g L-histidine/day for 3 days. The studies with L-histidine were carried out to determine whether increased histamine production could be achieved in patients with Raynaud's disease for use as a possible form of therapy.

Significance: The enzymatic assay of histamine in urine provides a simple method of detecting alterations in synthesis or metabolism of histamine by measuring changes in the excretion of histamine. The assay makes it possible to evaluate agents that enhance or inhibit histamine synthesis or metabolism. For example, the effect of an inhibitor of histidine decarboxylase in a patient with mastocytosis can be determined by measuring the decrease in urinary histamine.

Proposed Course of Project: The assay procedure will be utilized in studies of the release of histamine in various pathological states and in the *in vivo* evaluation of compounds that alter the metabolism of histamine.

Honors and Awards: None

Publications:

1. Horáková, Z., Zierdt, C. H., and Beaven, M. A.: Identification of Lactobacillus as the source of bacterial histidine decarboxylase in rat stomach. Europ. J. Pharmacol. In press.

Serial No. NHLI-89
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation and Characterization of Hydroxyindole O-methyl Transferase.

Previous Serial Number: None

Principal Investigator: Walter Lovenberg, Ph. D.

Other Investigators: Richard Jackson, Ph. D. and Joseph Fontana, Ph. D.

Project Description:

Objectives: The principle objective of this project was to obtain a pure preparation of hydroxyindole O-methyl transferase (HIOMT) and to study its physical and chemical properties.

Methods: The enzyme activity was assayed using ^{14}C-S-adenosyl methionine as the methyl donor and N-acetylserotonin as the acceptor. The product, melatonin, was extracted into an organic solvent and counted. The protein was purified by standard isolation techniques.

Major Findings: Bovine pineal HIOMT was isolated as an essentially homogenous protein. The steps developed for this isolation in good yield are summarized below:

	Specific Activity	% Yield
Crude extract	15	100
Ammonium sulfate	55	100
1st DEAE Sephadex	122	75
Sephadex G 100	200	43
2nd DEAE Sephadex	274	30
Isoelectric Focusing	343	29

The final product gave a single band on disc gel electrophoresis and appeared to be homogenous when studied by sedimentation equilibrium in an ultra-centrifuge. Antibodies to this apparently pure enzyme were prepared in sheep. Gel diffusion or gel electrophoresis of the protein and reaction with these antibodies also indicated that a single molecular species had been isolated. It is interesting to note that at least 3 molecular weight forms of the enzyme were observed on Sephadex G-100 chromatography and that chromatography of the major form on the 2nd DEAE Sephadex step resulted in the isolation of 2 differently charged enzymes. Each of these forms could be completely purified by isoelectric focusing. These two forms had identical immunologic

properties and appeared to be identical in all other respects. The molecular weight of the enzyme was about 78,000 as determined by sedimentation equilibrium. Electrophoresis in sodium dodecyl sulfate (SDS) containing gels indicated that the molecular weight was about 39,000. The native enzyme probably contains 2 subunits of about this molecular weight. Fingerprint analysis of tryptic digests of the protein showed that the two subunits were very similar or identical. The amino acid content was determined and was unique only in that this protein contained relatively high content of leucine (15-20). The enzyme did not appear to contain any sugar residues. Although, other phenolic O-methyl transferases require metal ions for activity, examination of a wide variety of metal ions and metal chelators indicate that metals do not participate in this methyl transfer reaction.

Significance: It was previously thought that HIOMT was the rate limiting enzyme in the biosynthesis of melatonin, and that diurnal changes in this enzyme were responsible for the corresponding changes in hormone levels. This concept has recently been questioned and from the current work in which we find that 4% of the total soluble pineal protein is HIOMT and that the enzyme has a low turnover number it would appear to be unlikely that this enzyme represents a control point in the pathway. The presence of a number of different molecular species suggest that possible conversion of one form to another may be a means of controlling enzyme activity *in vivo*, although there is yet no evidence for such a mechanism.

Proposed Course of Project: With the availability of large amounts of antibodies to this enzyme it should be possible to specifically study its biosynthesis. This type of experimentation is planned. We also plan to investigate the means by which the various molecular forms of the enzyme are formed or interconverted.

Publications: None

Serial No. NHLI-90
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1971

Project Title: A Sensitive and Automated Method for Recording Gastric pH Changes and H^+ Ion Secretion in Experimental Animals and the Use of this Method for Studying the Pharmacology of Gastric Secretion.

Previous Serial Number: NHLI-150

Principal Investigator: G. H. Besselaar, M.D.

Other Investigators: Ronald Geller, Ph. D. and Walter Lovenberg, Ph. D.

Project Description:

Objectives: 1) To develop a sensitive method for measuring pH and actual H^+ ion secretion in the stomach of experimental animals. 2) Use of this method for studying the effects of drug administration on gastric secretion.

Methods: The model for continuous recording of gastric secretion in the rat was described in last year's project report. The only modification was in the perfusate, which was changed from dilute base (.00025 N NaOH) to .9% saline pH 7.00. The endpoint of the titration unit was set at the pH of the inflowing saline, so that in effect the total amount of H^+ ions secreted by the stomach is neutralized. Gastric secretion in response to various pharmacological agents was studied in normal and vagotomized Sprague-Dawley rats (250-350 grams).

Major Findings: 1) Intravenous infusions of small doses of histamine (8 µg/min) and pentagastrin (.4 µg/min) consistently stimulate gastric secretion. These doses result in about half-maximum secretion in most animals. 2) For vagotomized animals (7-10 days post-operative) the response to the above mentioned doses of histamine and pentagastrin is either absent or very slight. With much higher doses, however, the peak response to histamine and pentagastrin is the same as in control animals. 3) Serotonin (3 µg/min i.v.) infusions have an inhibitory effect on gastric acid output stimulated by histamine (8 µg/min). This effect cannot be demonstrated when gastric secretion is maximally stimulated by histamine, whereas the results of similar experiments using pentagastrin as the stimulant are variable. 4) L-Tryptophan and 5HTP in acute experiments had no effect on histamine or pentagastrin stimulated gastric secretion. 5) Pretreatment with parachlorophenylalanine (PCPA 300 mg/kg for 3 days), a tryptophan hydroxylase inhibitor, resulting in serotonin depletion in the proximal duodenum, and a concomitant increase in gastric secretion in Shay rats. Perfused rats pretreated with PCPA showed a decreased sensitivity to histamine and to serotonin. Further work along

these lines is in progress. 6) We have been unable to demonstrate an inhibitory effect of secretin on either histamine or gastric stimulated secretion, using dose levels similar to those used in man and dogs where the inhibitory effect has been observed. 7) Norepinephrine, angiotensin, pitressin and PGE, which have been shown in the dog to decrease gastric mucosal bloodflow, all are strong inhibitors of gastric secretion in the perfused rat stomach.

Significance: There is good evidence for a duodenal mechanism controlling gastric acid secretion. Acidification of the duodenum leads to inhibition of gastric secretion, and to release of serotonin and secretin from this area. Both compounds, under various circumstances, have been shown to be able to inhibit histamine and/or gastric stimulated secretion. Depletion of endogenous serotonin leads to increased secretion; administration of exogenous serotonin results in inhibition of gastric secretion. By expanding our data with PCPA (and reserpine) pretreated animals and studying the effect of vagotomy in these animals we hope to clarify the possible physiologic importance of serotonin in the duodenal inhibitory mechanism of gastric acid secretion.

Proposed Course of Project: Expansion of the studies of the effect of PCPA and reserpine on gastric acid secretion in normal and vagotomized animals is planned. Studies on the effect of 5HTP pretreatment on gastric secretion on normal and vagotomized animals will also be carried out.

Publications: None

Serial No. NHLI-91
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tryptophan Hydroxylase and Serotonin in Spinal Cord and Brainstem Before and After Chronic Transection.

Previous Serial Number: NHLI-145

Principal Investigator: B.V. Clineschmidt, Ph. D.

Other Investigators: Walter Lovenberg, Ph. D. and J.E. Pierce, D.V.M.

Cooperating Units: Section on Laboratory Animal Medicine & Surgery, NHLI

Project Description:

Objectives: 1) To ascertain whether the spinal cord of the cat contains serotonergic interneurones and 2) to compare the ratio of tryptophan hydroxylase activity to serotonin (5HT) content in the area rich in serotonin-containing cell bodies (brainstem) with an area containing predominately axons (spinal cord), many centimeters removed from their cell bodies.

Findings: Tryptophan hydroxylase and serotonin levels were determined in normal and chronically transected (T_8-T_9) cats. Lumbosacral and brainstem 5-HT and tryptophan hydroxylase activity were:

	5-HT µg/gm		Tryptophan Hydroxylase mµmoles g^{-1} hr^{-1}	
	cord	brainstem	cord	brainstem
Control	0.70	0.94	8.4	13.0
3 week transection	0.10	1.19	1.8	15.8
5 week transection	0.11	1.04	1.4	18.0

Both 5-HT and tryptophan hydroxylase were decreased 80 to 85% in the cord of animals transected for 3 weeks but significant amounts appeared to remain. Extending the duration of transection from 3 weeks to 5 weeks caused no further decrease in either parameter. Brainstem 5-HT was not affected by spinal transection. However, 5 weeks after transection brainstem tryptophan hydroxylase was significantly increased.

Significance: 1) 5-HT Containing interneurones. According to workers employing the histochemical fluorescent technique, all 5-HT in spinal cord resides in axons from neurones whose cell bodies lie in the Raphe nuclei of the brainstem. On the other hand, recent neuropharmacological evidence

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indicates that spinal cord may contain serotonergic interneurones. If all spinal 5-HT originates supraspinally, cords removed below the level of transection from chronically transected animals should contain insignificant amounts of tryptophan hydroxylase (19% of normal) and 5-HT (14% of normal) after complete degeneration of descending axons. These residual levels may reflect the existence of serotonergic interneurones in spinal cord.

2. Tryptophan hydroxylase-5-HT ratio in cell bodies vs. axons. It was suggested recently that the brainstem level of tryptophan hydroxylase exceeds the amount required to maintain the steady-state level of 5-HT, perhaps because the brainstem contains mostly cell bodies where the enzyme presumably is synthesized. We found no difference in the ratio of tryptophan hydroxylase to 5-HT in brainstem (predominately cell body area) as compared with spinal cord (predominately axon area). Thus, it might be proposed that the level of tryptophan hydroxylase determines the concentration of 5-HT in a given area of the brain regardless of whether the area contains mainly cell bodies or axons. However, such a proposal is difficult to reconcile with the increase in tryptophan hydroxylase without an accompanying rise in 5-HT which we observed in brainstem after chronic spinal transection.

Proposed Course of Project: None

Publications:

1. Clineschmidt, B.V., Pierce, J.E. and Lovenberg, W.: Tryptophan hydroxylase and serotonin in spinal cord and brainstem before and after chronic transection. J. Neuro. Chem. In Press.

Serial No. NHLI-92
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Biological Role of Histamine: Participation of Histamine in Inflammation Following Heat Injury

Previous Serial Number: None

Principal Investigator: Zdenka Horáková

Other Investigators: Michael A. Beaven, Ph.D.

Cooperating Units: None

Project Description:

Objectives: In a continuing study of the role of histamine in the body, the release of histamine in inflammation following heat injury was examined. These studies were undertaken with the aim of reinvestigating the participation of histamine in the inflammatory response to heat injury. The current opinion holds that the role of histamine, if any, in the development of inflammation is transient and that other mediators are necessary to maintain inflammation. This opinion is based upon the finding that histamine is released from injured tissues at a rapid rate and that it has largely disappeared from tissue at a time when inflammation is still developing. However, a limitation in these experiments is that the assays were insufficiently sensitive to measure low levels of histamine that may be present after depletion of histamine stores and which may represent newly synthesized histamine. The availability of the specific and sensitive enzymatic assay for histamine makes it possible to obtain more precise information on the changes in tissue histamine during the course of the inflammatory response to injury.

Methods: Heat injury was produced in the rat paw by immersion of the paw in hot water (56°C) for 30 sec. At various time intervals after injury, samples of the skin and underlying tissue were removed from the paw by skin-punch. Samples of wound exudate and aortic blood were also collected. The amount of edema was assessed by measurement of paw volume. Histamine was assayed by the enzymatic procedure described in Project Report NHLI-144, 1969-70.

Major Findings: The following observations were made after heat injury:

1) Edema of the rat paw was evident within a few minutes of heat injury and by one hour the paw had swollen to 2 to 3 times its normal size. The edema persisted for the remaining 24 hours of the experiment.

2) There was a transient increase in histamine levels in the paw skin immediately following heat injury. This increase persisted for a few minutes and was followed by a rapid reduction in the histamine levels. Twenty minutes after the heat injury, histamine levels in the paw skin were depleted by 70% compared to control and remained depleted for 24 hours.

3) Large amounts of histamine appeared in the tissue exudate after heat injury. In non-heat injured animals, the normal exudate contained 300-400 ng histamine/ml exudate. In heat injured animals, the histamine content of the exudate rose to 2-3,000 ng/ml by 20 min and remained elevated, 1,500 ng/ml, at one hr after heat injury. The histamine levels in the exudate slowly subsided during the 24 hr of the experiment but were still high, 500 ng/ml, compared to levels in non-heat injured animals, 24 hr after injury. Histamine levels in the circulating blood were slightly increased after heat injury.

4) In animals that were depleted of tissue histamine by administration of the histamine liberator, compound 48/80 (1 mg/kg, i.p., four times over 48 hr), heat injury resulted in only a small increase, 30%, in paw volume compared to a 2 to 3-fold increase in non-48/80 treated rats. The amount of histamine in the wound exudate of the 48/80 treated animals was less than 5% of that seen in nontreated rats after heat injury.

Significance: The application of heat injury to the rat paw provides a useful model for studying the role of histamine during inflammation. The studies show the increased levels of histamine persisted in tissue fluid for up to 24 hr after heat injury. The amount of histamine present could be sufficient to account for the edema in the injured paw and is evidence that the action of histamine in inflammation is of long duration. Correspondingly, in animals that were depleted of tissue histamine, the edematous response to heat injury was only 10% of that of normal injured animals. These findings are important in the consideration of the mechanism of edema formation and will be the basis of future investigations of vascular responses to heat injury.

Proposed Course of Project: Studies will be continued to determine whether the elevated histamine levels in tissue fluids after heat injury represent newly synthesized histamine or histamine released from tissue stores. The investigations will include studies of mild heat treatment (48°C) in which responses such as vascular dilation and edema are reversible. The effect of agents which promote histamine synthesis or block its metabolism and the effect of antiinflammatory agents on the response to histamine during heat injury will be studied. It is hoped that such investigations will provide information on whether histamine plays a role in vascular physiology.

Honors and Awards: None

Publications: None

Serial No. NHLI-93
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Mechanisms of Binding and Release of Biogenic Amines.

Previous Serial Number: NHLI-148

Principal Investigator: Larry Loeffler, Ph. D.

Other Investigators: Walter Lovenberg, Ph. D.

Cooperating Units: None

Project Description:

Objectives: To further elucidate the manner in which biogenic amines are bound in cellular storage sites and the mechanism by which release occurs in response to various chemical agents. In particular, to study the mechanism of histamine release from mast cells.

Methods: The peritoneal mast cell system of the rat has been used to study: 1) the release of histamine by the selective releasing agent compound 48/80 or high molecular weight dextran; 2) the effects of certain adenine nucleotides, phosphodiesterase inhibitors and prostaglandin E_1 upon 48/80 induced histamine release; 3) the binding of tritium labelled 48/80 to mast cells; and 4) the nature of the protein material in storage granules of the mast cell. In vitro cell suspensions and well established assay procedures have been employed in all of these studies.

Major Findings: 1) Histamine release studies and 2) The effects of adenine-nucleotides, phosphodiesterase inhibitors and prostaglandin E_1. Heterogeneous peritoneal cell suspensions were found to respond uniformly and consistently to compound 48/80 (concentration of about 1 µg/ml for 5 minutes) as described in the literature, giving a very rapid release of 70-80% of the total histamine content. Preincubation of the cell suspensions for short time (i.e. 20 min) with dibutyryl-3'5'-c-AMP (DB-c-AMP) was found to cause inhibition of histamine release by compound 48/80. Strong inhibition was observed at 10^{-2}M concentration, statistically significant inhibition at 10^{-3}M. The following adenine nucleotides were found to be ineffective as inhibitors at similar concentrations: 5'-AMP; 5'-ADP; 5'-ATP; and 3'5'-c-AMP. Adenosine, phosphate and pyrophosphate were also ineffective.

In an effort to obtain effects with DB-c-AMP at concentrations approaching physiological, inhibitors of c-AMP phosphodiesterase were studied and were also found to inhibit 48/80 induced release, even in the absence of added DB-c-AMP. Theophylline was completely inhibitory at 10^{-2}M concentration and partially effective at 10^{-3}M. The phosphodiesterase inhibitors reserpine, DEAE-reserpine and perphenazine were very significantly inhibitory at concentrations of 3×10^{-4}M, 5×10^{-5}M and 6×10^{-5}M, respectively. These concentration levels are similar to those required for phosphodiesterase inhibition. The latter three inhibitors, but not theophylline, themselves promoted a less dramatic release of histamine compared with 48/80 during prolonged incubation periods. The inhibitory effects of theophylline or DB-c-AMP could be removed by washing of centrifuged cells and the response of resuspended cells to the releasing agent 48/80 nearly completely restored. Prostaglandin E_1, which would be expected to stimulate adenyl cyclase and elevate c-AMP levels, was also found to be an effective histamine release inhibitor, at concentrations of 3×10^{-5}M and above.

The above interest in the in vitro effects of DB-c-AMP and theophylline was stimulated by the work of Drs. H. H. Baxter, M.A. Beaven and Z. Horakova, who found that these agents protect rats against the anaphylactoid response to dextran and effectively block the normal elevation of blood histamine found after administration of dextran.

3) Binding of tritium labelled 48/80 to mast cells. Compound 48/80 was randomly labelled with tritium via the Wilzbach procedure and the product was found to retain its histamine releasing activity. By means of column chromatography on CM Sephadex and Sephadex, four major peaks of radioactivity were obtained. All of the biological activity resided in the major or highest molecular weight fraction. At a concentration of 1 µg/ml, only about 3-4% of the labelled material became "bound" to the cells after incubation. No significant differences in binding were observed upon preincubation of the cells with the release inhibitors DB-c-AMP and theophylline. Therefore, these experiments offer no evidence that these compounds inhibit histamine release by inhibiting the binding of 48/80.

4) Investigations of the nature of the protein component of the mast cell storage granule. Homogenous mast cells, obtained by density gradient centrifugation of peritoneal cell mixtures, were ruptured by sonication and the granules isolated by differential centrifugation. Disc gel electrophoresis studies on the granular material (heparin-protein-histamine complex) indicated that the protein material was a complex multicomponent one, and no further work was initiated.

Significance: Previously reported studies indicate that release of histamine from mast cells by 48/80 involves: 1) attachment of 48/80 to the cell membrane, 2) specific and extremely rapid degranulation by an energy requiring "exocytosis" of granules which contain a histamine-heparin-protein complex, and 3) liberation of the heparin bound histamine from the granules by an ion exchange process with cations of the extracellular fluid. The effects of DB-c-AMP, various phosphodiesterase inhibitors and prostaglandin E on this

release process can be interpreted as circumstantial evidence supporting a role for the adenyl cyclase system. Higher levels of cyclic AMP would appear to protect the cell against the effects of 48/80, perhaps by altering the energy requiring release process. Elevated levels of c-AMP can be promoted by DB-c-AMP which presumably enters the cell rapidly, phosphodiesterase inhibitors, which prevent the destruction of c-AMP and PGE_1, which stimulates adenyl cyclase. Definitive biochemical studies on the adenyl cyclase system of homogeneous mast cells and measurement of c-AMP levels in response to release inhibitors are required to establish definitely the role of the adenyl cyclase system if any.

Proposed Course of Project: No further work is contemplated. Extension of these observations to antigen-antibody promoted histamine release, and definitive biochemical studies of the adenyl cyclase system in the mast cell, would be of considerable interest in the understanding of allergic responses.

Publications:

1. Loeffler, L.J., Lovenberg, W., and Sjoerdsma, A.: Effects of dibutyryl-3'5'-cyclic-adenosine monophosphate, phosphodiesterase inhibitors and prostaglandin E_1 on histamine release from rat peritoneal mast cells *in vitro*. Biochemical Pharmacol. In Press.

Serial No. NHLI-94(c)
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Biosynthesis and Metabolism of Physiologically Active Amines.

Previous Serial Number: NHLI 149

Principal Investigator: Walter M. Lovenberg, Ph.D.

Other Investigators: B. Van Clineschmidt, Ph.D., Paul J. Schechter, M.D., Ph.D., David Horwitz, M.D., and Richard Wyatt, M.D.

Cooperating Units: Adult Psychiatry Branch, National Institute of Mental Health.

Project Description:

Objectives: A number of phenylethyl- and indolealkylamines have physiological roles or are pharmacologically active. The objectives of this study are to learn more about the physiologic roles of amines and to understand what controls their synthesis and degradation. The studies are directed specifically towards serotonin, dopamine, and norepinephrine.

It has been reported recently that morphine addiction in mice results in an increased synthesis of serotonin in the brain. In this study this finding is reexamined and the rate limiting enzyme (tryptophan hydroxylase) in serotonin synthesis is measured in morphine addicted mice. The effect of chronic administration of a serotonin antagonist (methysergide) or a serotonin depletor (reserpine) on tryptophan hydroxylase in the brain is measured in an effort to further understand the mechanisms controlling serotonin synthesis. In addition, the levels of tryptophan hydroxylase have been measured in REM deprived rats. Finally, the level of dopamine-β-hydroxylase has been examined in normal and hypertensive human serum.

Methods: Mice were addicted to morphine by subcutaneous implantation of a morphine pellet (75 mg) for 3 days. Rats were treated with reserpine 2.5 mg/kg daily for 3 days or with methysergide 5 mg/kg twice daily for 5 days. Serotonin synthetic rates in mice were measured by the rate of accumulation of serotonin in the brain following administration of a monoamine oxidase inhibitor. Tryptophan hydroxylase was measured in the supernatant fraction of brain homogenates using a tritium release assay developed in this laboratory. Deprivation of REM in rats was achieved by confining the animals to 6 cm flower pots inverted in containers of tepid water.

Major Findings: The tryptophan hydroxylase activity in the brain of mice addicted to morphine (95 ± 9 μμmoles/mg) showed no significant difference from mice implanted with control pellets (102 ± 7 μμmoles/mg/hr). The adequacy of the addiction treatment was confirmed by measuring Naloxone withdrawal (jumping test). Since there was no difference in the rate controlling enzyme, the original observation on increased brain serotonin synthesis in addicted mice was reinvestigated. In contrast to the reports in the literature, we find no difference in the rate of serotonin accumulation following a monoamine oxidase inhibitor in morphine addicted animals.

Rats treated chronically with reserpine have brainstem tryptophan hydroxylase levels which are identical with normal rats. The brainstem tryptophan hydroxylase levels in rats treated with methysergide, however, appeared to be slightly lower than controls (0.25 vs 0.35 mμmoles/mg/hr) in preliminary experiments. It was expected that tryptophan hydroxylase activity might be increased following REM deprivation since it has been reported that serotonin turnover increases. In this study, however, the REM deprived animals did not show a significant difference from the appropriate control animals (0.27 vs 0.31 mμmoles/mg/hr). The stressed control animals (large pots surrounded by water) showed significantly higher tryptophan hydroxylase than nonstressed controls (0.23 vs 0.31 mμmole/mg/hr).

Since the enzyme dopamine-β-hydroxylase is released into plasma during sympathetic activity and one of the factors in some types of human hypertension is increased "sympathetic" activity, a study measuring dopamine-β-hydroxylase in control and hypertensive patients was started. Normal levels of enzyme activity were found to range from 130 to 500 mμmoles of tyramine hydroxylated per 20 minutes per ml of plasma. Two patients with pheochromocytoma and 2 hypertensives had levels in this range. A third patient with hypertension had a very low level of this enzyme (2-4 mμmoles tyramine/ml/20 min).

Significance: The increasing use of psychoactive drugs medically and abuse of these compounds by the general population makes it imperative that we understand the mechanism by which these drugs act. Many of these compounds appear to relate in one way or another to serotonin metabolism in the central nervous system. Although the results are largely negative they are of value to our understanding of a relatively unknown aspect of the mechanism of action of psychoactive drugs. The studies in the REM deprived animals do little to further our understanding of the role of serotonin in REM sleep. They do provide evidence that tryptophan hydroxylase levels are not necessarily the controlling factor in serotonin turnover.

Proposed Course of Project: Studies will be continued on the possible decrease in tryptophan hydroxylase after methysergide treatment, and the effect of other serotonin antagonists will be evaluated. A much wider study on the dopamine-β-hydroxylase in plasma is planned. The patient with the extremely low levels of this enzyme will be further studied to determine if the essential absence of dopamine-β-hydroxylase is in any way related to his hypertension.

Honors and Awards: None

Publications:

1. Lovenberg, W. and Engelman, K.: Serotonin. In: Handbook of Clinical Laboratory Data (Ed. 3). Cleveland, Ohio, Chemical Rubber Co. In press.

2. Lovenberg, W.: Some vaso- and psychoactive substances in food - amines, stimulants, depressants and hallucinogens. In: Toxicants Occurring Naturally in Foods. Washington, D.C. National Research Council Publication. In press.

Serial No. NHLI-95
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Shock-induced Aggression in Hypertensive Rats.

Previous Serial Number: None

Principal Investigator: Wybren DeJong, M.D., Ph. D.

Other Investigators: B. Eichelman, M.D., Ph. D. (LCP-NIMH) and R. B. Williams, M.D. (LCP-NIMH).

Cooperating Unit: Laboratory of Clinical Psychobiology, National Institute of Mental Health.

Project Description:

Objectives: Behaviour is an important aspect of hypertension and little information is available on the behaviour of hypertensive rats. We felt that a study of aggression in the genetically pure strain of spontaneously hypertensive rats (SHR) might give valuable data when compared to adequate normotensive and hypertensive controls.

Methods: Fighting was induced in paired female rats by applying 50 electric shocks (2mA) to their feet in each session on four consecutive days. In addition mouse killing was studied in all rats used and we also determined the jump-flinch thresholds by a standardized method. The rats used were 10-12 weeks old. Renal hypertension was induced by application of a solid silver clip (0.25 mm int. diameter) on the left renal artery in 8 weeks old rats. These rats were studied 5-7 weeks after the operation.

Major Findings: In normotensive Wistar rats (NIH) a high fighting rate was observed on all four days, while another normotensive strain (Sprague-Dawley rats, Zivic Miller Laboratories) showed low fighting rates on all four days. Spontaneously hypertensive rats had a high fighting rate on the first day studied which decreased to low levels on the subsequent days. Comparison to renal hypertensive rats (Wistar strain, NIH) indicated that this decrease was not a direct effect of the high blood pressure since fighting rates of the renal hypertensive rats remained high as observed in normotensive Wistar controls. No significant differences in mouse killing were observed between the different groups of rats studied. A slightly lower jump-flinch threshold was observed in the S.H. rats.

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Significance: These findings show that high blood pressure by itself does not affect shock-induced fighting of paired rats. The decrease of fighting rates of SHR may be specific for genetic hypertensive rats.

Proposed Course of Project: Shock-induced aggression in another type of genetic hypertensive rats (salt dependent hypertension) will be studied to obtain information about the specificity of the observed decrease in fighting rate in SHR.

Publications: None

Serial No. NHLI-96
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Renin-angiotensin System of the Spontaneously Hypertensive Rat.

Previous Serial Number: None

Principal Investigator: Wybren De Jong, M.D., Ph.D. (Guest Worker)

Other Investigators: Walter M. Lovenberg, Ph.D. and Albert Sjoerdsma, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: The renin-angiotensin system is of importance in hypertension and its role is insufficiently documented in the genetically pure strain of spontaneously hypertensive rats (SHR). To obtain a greater understanding of the contribution of this system to the high blood pressure in these animals we investigated this system in male SHR at different ages.

Major Findings: Plasma renin activity of SHR (3.9 ng/ml) was found to be higher than in controls (1.5 ng/ml) at 12 weeks of age and this difference remained up to 35 weeks of age. At 8 weeks in the initial experiment no significant difference occurred. However, in additional experiments an increase of plasma renin activity of SHR was found as early as 6 weeks of age. An increase in plasma renin substrate was observed in SHR of 16 weeks and older. At the age of 20 weeks renin substrate levels were 665 + 18 ng/ml in Wistars and 905 + 43 ng/ml in the SHR. A marked decrease in kidney renin content was found in SH rats at 35 weeks of age. When renal renin release was stimulated by pentobarbital, plasma renin activity of renal venous blood of SHR did not differ from that of normotensive Wistar rats. Also no difference in plasma renin activity was observed when measured at the time of peak activity during normal diurnal rhythm. Preliminary studies indicated that the elevated plasma renin in SHR may arise from an extrarenal source, since it is still present in plasma of these rats 18 hours after nephrectomy.

Significance: Several investigators suggested that the renin angiotensin system is depressed by the high blood pressure level in genetic hypertensive rats. The decrease of kidney renin content in older animals might be a reflection of such a mechanism. The present findings, however, do not confirm such a mechanism in SH rats. The significance of the observed increased plasma renin activity in SHR for the high blood pressure remains to be ascertained. Occurrence of a extrarenal renin-like enzyme that contributes to

plasma renin activity is unusual but has been shown in nephrectomized patients and in different animal species after a bilateral nephrectomy.

Proposed Course of Project: It is proposed to clarify the mechanism which induces increased plasma renin activity in SH rats, to determine the source of the presumably extra-renal renin like enzyme and to evaluate the contribution of the increased plasma renin activity to the hypertension.

Honors and Awards: None

Publications: None

Serial No. NHLI-97
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Tryptophan Hydroxylase and Phenylalanine Hydroxylase: Comparative Properties, Purification and Interactions.

Previous Serial Number: None

Principal Investigator: Richard E. Bensinger, M.D.

Other Investigators: Walter M. Lovenberg, Ph.D.

Cooperating Units: None

Project Description:

Objectives: Tryptophan hydroxylase and phenylalanine hydroxylase catalyze an important step in the biogenesis of clinically significant intermediary substances in amine metabolism. The fundamental properties appear similar for the two enzymes but little work has been done comparing the two. This project will investigate these interrelationships and attempt to purify the enzymes to accomplish this purpose. In addition, clinically useful information related to diagnosis of disorders involving these two enzymes is being sought.

Major Findings: A new assay for tryptophan hydroxylase has been developed. This assay is based on the release of tritium atoms from 5-^{3}H-tryptophan following hydroxylation. Although the "NIH shift" occurs, incubation of the product, 4-^{3}H-5-hydroxytryptophan, under acid conditions results in complete release of the tritium. The assay can measure the formation of as little as 0.2 mμmoles of 5-hydroxytryptophan and can be used with small tissue samples from the central nervous system, liver, or kidney. This assay and a similar one for phenylalanine hydroxylase has facilitated the remainder of the studies.

Tryptophan hydroxylase from bovine pineal has been purified some 30-fold using standard protein fractionation procedures. Phenylalanine hydroxylase has also been partially purified from rat liver by a published procedure. Attempts to more easily prepare this enzyme using affinity chromatography resulted in the isolation of a fraction from rat liver which had very high specific activity using phenylalanine as a substrate, but little activity toward tryptophan. This fraction, however, strongly stimulated tryptophan hydroxylase which had been partially purified from bovine pineals. The tryptophan hydroxylase stimulating factor (THS) was present in crude tissues and appeared to be purified simultaneously with phenylalanine hydroxylase activity. It was both heat labile and non-dialyzable. It is possible that THS is

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synonomous with liver phenylalanine hydroxylase. THS both stimulates tryptophan hydroxylase and shifts the pH optimum from 7.3 to 8.2. Although the partially purified fractions from beef pineal hydroxylate both phenylalanine and tryptophan, only tryptophan hydroxylating activity is stimulated. Studies on cofactor specificity indicate that only ferrous iron satisfies the metal requirement and that ascorbic acid will not replace the reduced pteridine requirement.

Another part of this project is designed to develop specific radiopharmaceuticals which will localize tumors having an excess of these enzymes, or enzymes of related systems. The labeled nuclide is attached to substrates or inhibitors having a high affinity (low K_m or K_i) for the enzyme, thus binding there and identifying the neoplastic tissue. Preliminary experiments are being conducted by measuring the tissue distribution of 131-iodotyrosine in rats. Since this is an inhibitor with high affinity for tyrosine hydroxylase, it might be expected to be differentially retained by the adrenal glands which have a large amount of this enzyme. No such distribution is yet evident.

Significance: The development of a simple and sensitive radioassay for tryptophan hydroxylase will greatly enlarge the scope of the work that can be done with this important enzyme. The finding of a second protein factor that stimulates tryptophan hydroxylase may in part explain the great difficulties previously encountered in attempts to purify this enzyme. Since the stimulating factor appears to be identical with phenylalanine hydroxylase, it suggests that mammalian hydroxylating enzymes may consist of several subunits and that the substrate specificity in individual organs may reflect a particular combination of these units. These findings also have implications for the disease phenylketonuria in which individuals lack the enzyme phenylalanine hydroxylase but appear to be capable of forming hydroxyindoles. Further understanding of the hydroxylase reactions are important in the design of compounds to help regulate serotonin synthesis in mental disorders and in hydroxyindole producing tumors (carcinoid).

The development of techniques using radionuclides to detect areas of relatively high concentration of certain enzymes would be of obvious benefit both in the diagnosis and surgical removal of tumors.

Proposed Course of Project: The complete isolation of the tryptophan hydroxylase stimulating factor will be attempted. Using this factor attempts to isolate pineal tryptophan hydroxylase will be made. Continued attention will be devoted to possible inhibitors and their possible clinical significance.

Honors and Awards: None

Publications:

1. Lovenberg, W. and Jackson, R.L.: Tryptophan-5-hydroxylase. In: Methods in Investigative and Diagnostic Endocrinology. In press.

2. Lovenberg, W., Bensinger, R.E., Jackson, R.L., and Daly, J.W.: Rapid analysis of tryptophan hydroxylase in rat tissue using 5-^{3}H-tryptophan. Anal. Biochem. In press.

Serial No. NHLI-98
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Isolation and Characterization of Clostridial Electron Transfer Proteins and Other Iron-Sulfur Proteins.

Previous Serial Number: NHLI-154

Principal Investigator: Walter M. Lovenberg, Ph.D.

Other Investigators: William A. Eaton, M.D., Ph.D.

Cooperating Units: Laboratory of Physical Biology, NIAMD; Central Research Department of DuPont, Wilmington, Delaware; Department of Chemistry, Pennsylvania State University, University Park, Pennsylvania.

Project Description:

Objectives: Iron-sulfur proteins are a large class of proteins that catalyze many vital redox functions in living organisms. The major objectives of this project are to elucidate the structure of the active center of this type of protein, and to learn how this relates to its role in accepting or donating electrons. Both rubredoxin, a single-iron protein, and the ferredoxin type multi-iron proteins have been used in this work. Clostridial rubredoxin which was first isolated in this laboratory several years ago is the most simple of the iron-sulfur proteins, consisting of a single polypeptide chain which contains four cysteine residues, positioned in such a way that the sulfhydryl groups tightly chelate a single iron atom. The ferredoxin type protein also consist of a single polypeptide, but these contain several sulfhydryl groups which form a much more complex structure with 2 or more iron and inorganic sulfide atoms. These proteins normally occur in the oxidized form and the addition of one or two electrons to the iron-sulfur complex results in changes in the physical-chemical properties that provide clues for determining the nature of the active center. Several chemical and physical techniques have been used to further understand the nature of the iron binding site.

Methods: A. Rubredoxin. Rubredoxin from Clostridium pasteurianum consists of 54 amino acids. Using the classical amino acid sequencing techniques and data provided by x-ray crystallographic analysis the following tentative sequence has been determined:

F-Met Lys Lys Tyr Thr Cys Thr Val Cys Gly Tyr Ilu Tyr Asp Pro Glu Asx Gly Asx

Pro Val Asx Gly Asx Asx Asp Gly Thr Asp Phe Lys Asp Ilu Pro Asp Asp Try Val

Cys Pro Leu Cys Gly Val Gly Lys Asp Glu Phe Glu Glu Val Gln Glu

This sequence shows a number of homologies with the 2 other rubredoxins from anaerobes that have been sequenced, and with each half of the much larger Pseudomonad oleovoran rubredoxin. Most important, however, is the observation that the cysteine residues occur in homologous positions since it is the sulfhydryl groups that hold the iron atom. Also of interest is the finding that the initiator amino acid of bacterial polypeptide synthesis, N formylmethionine, is retained at the N terminus. This is the first native protein that has been found to retain this residue.

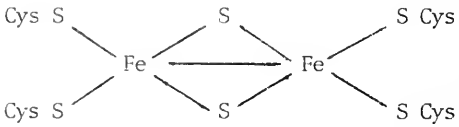
The geometry of the ligand field of the high spin iron atom in rubredoxin was reported previously to be approximately tetrahedral. This was determined by x-ray crystallographic analysis, and by the measurement of a very optically active d → d transition in the near infrared using the technique for measuring circular dichroism in the infrared developed in this project. This work has been continued and extended. The original observations indicated an optically active absorption band at about 1.6 μ in reduced rubredoxin. Because of a "window" in the opacity of D_2O, we now have been able to extend the observed spectra to include 0.8 to 1.8 μ and 2.1 to 2.5 μ. The data indicate that another optically active band in reduced rubredoxin is centered at a somewhat lower energy than 2.5 μ. This band is also interpreted as arising from a d → d transition in the reduced rubredoxin. The splitting of the d → d absorption bands probably results from a distortion in perfect tetrahedral symmetry around the iron atom. This distortion has been confirmed by the large degree of dichroism in the visible absorption spectra of single crystals observed parallel and perpendicular to the crystal axis. The x-ray analysis also suggests some distortion. Another approach to the ligand geometry of rubredoxin has been examination of the Laser-Raman spectra in collaboration with Dr. Thomas V. Long. Excitation of the molecule with the 488 nm line of an Ar^+ laser results in Raman spectra which are consistent with a tetrahedrally bound iron atom.

Examination of the magnetic susceptibility, the nuclear magnetic resonance spectra and the Mössbauer spectra of rubredoxin all were consistent with the following conclusions: 1) the iron of rubredoxin is high spin ferric and high spin ferrous in the oxidized and reduced forms respectively, and 2) the iron is held in a ligand field consisting of the four cysteinyl sulfhydryls.

B. Ferredoxin Type Proteins. It was reported previously that in C. pasteurianum ferredoxin all the β protons of the cysteinyl residues were very strikingly contact shifted in the NMR spectrum. This was interpreted as resulting from the bonding of the sulfhydryl groups to the iron atoms since

these contact shifted resonances exhibited anti-Curie law behavior. Studies on this phenomenon have been further refined and extended over broader temperature ranges, but with no new fundamental discoveries.

More significant, however, has been the application of the techniques for measuring infrared absorption and circular dichroism developed in the rubredoxin study to plant ferredoxin and adrenodoxin. These two electron carriers which serve key roles in plant and mammalian metabolism each have 2 iron and 2 inorganic sulfide atoms and are similar in many physical properties, although the structure of their active centers is not known. The following proteins were examined: spinach ferredoxin, parsley ferredoxin and beef adrenodoxin. Each of these proteins had an optically active band at approximately 1.6 μ when in the reduced state. Although the extinction coefficient and anisotropy factor were not identical to rubredoxin, these bands were qualitatively similar and suggested the possibility that a high spin ferrous atom was tetrahedrally coordinated. Like rubredoxin these proteins also had absorption bands at energies lower than 2.2 μ. While the evidence is somewhat less convincing than in the case of rubredoxin, it is consistent with a recently proposed model for the active center of the 2 iron-2 sulfur protein depicted in 2 dimensions:



Significance: The development of indirect techniques using the simple iron-sulfur protein rubredoxin for probing the nature of the iron binding in other iron-sulfur proteins has been successful. Use of such techniques has facilitated a greater understanding of the active center in the very important iron-sulfur protein plant ferredoxin and adrenodoxin.

Proposed Course of Project: Work will continue on the use of infrared absorption and circular dichroism to elucidate the structure of the more complex iron-sulfur proteins. Studies on the role of various amino acid side chains in maintaining the conformation necessary for iron binding will be initiated. The single crystal spectra of rubredoxin will be examined carefully in an attempt to use this means to define the nature of the distortion in the tetrahedral ligand field.

Honors and Awards: None

Publications:

1. Phillips, W.D., Poe, M., Weiher, J.F., McDonald, C.C., and Lovenberg, W.: Proton magnetic resonance, magnetic susceptibility and Mössbauer studies of Clostridium pasteurianum rubredoxin. Nature 227: 574-577, 1970.

2. McCarthy, K. F. and Lovenberg, W.: N-formylmethionine: The N terminus of Clostridium pasteurianum rubredoxin. Biochem. Biophys. Res. Commun. 40: 1053-1057, 1970.

3. Eaton, W. A. and Lovenberg, W.: Near-infrared circular dichroism of an iron-sulfur protein. d → d transitions in rubredoxin. J. Am. Chem. Soc. 92: 7195-7198, 1970.

4. Long, T. V., II, Loehr, T. M., Allkins, J.R., and Lovenberg, W.: The determination of iron coordination in nonheme iron proteins using Laser-Raman spectroscopy. II. Clostridium pasteurianum rubredoxin in aqueous solution. J. Am. Chem. Soc. In press.

Serial No. NHLI-99
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Catecholamine Metabolism and Blood Pressure of Spontaneously Hypertensive Rats.

Previous Serial Number: NHLI-156

Principal Investigator: Hirohiko Yamabe, M.D.

Other Investigators: Wybren DeJong, M.D., Ph. D., B. Van Clineschmidt, Ph.D., Yukio Yamori, M.D., Walter Lovenberg, Ph. D. and Albert Sjoerdsma, M.D., Ph. D.

Cooperating Units: None

Project Description:

Objectives: Previous studies have shown that a genetically pure strain of spontaneously hypertensive rats (SHR) had lower levels of brainstem norepinephrine (NE) than similar Wistar control animals. The objective of this study was to attempt to correlate the reduced brainstem NE with the development of hypertension.

Major Findings: Norepinephrine Synthesis. The mechanism which results in decreased brainstem NE in the SHR is unknown. An examination of the enzymes responsible for the biosynthesis of NE in SHR and control rats indicated that tyrosine hydroxylase was similar in each, but the level of aromatic L amino acid decarboxylase was reduced to about 50% in SHR. The decrease in decarboxylase was apparent even in newly born animals. Crosses between SHR and control animals gave a F_1 generation that had decarboxylase levels that were intermediate between the hypertensive and control animals. Examination of the kinetic properties of the enzyme from each source revealed that the K_m values for L-5-hydroxytryptophan and L-dopa were essentially the same from either strain. The values for L-5-HTP were about 8×10^{-6}M and for L-dopa 2.5×10^{-4}M. Thus, it appears that the gross amounts of aromatic L-amino acid decarboxylase in these two strains of Wistar rats are different and that these levels are genetically controlled.

Effects of 6-Hydroxydopamine (6-HDA). It is known that 6-HDA destroys sympathetic nerve endings when administered peripherally. If the hypertension in the SHR was in any way related to over-activity of the sympathetic nervous system, then destruction of the peripheral sympathetic nerves should result in a reduction in blood pressure. Administration of 6-HDA resulted in

destruction of up to 80% of the sympathetic nerves in heart, spleen, and kidney as determined by catecholamine content of these organs. The treatment resulted in no more than a very transient effect on blood pressure. Conversely it was felt that if levels of catecholamines in the brainstem were inversely related to blood pressure that central administration of 6-HDA in normal animals should result in the development of hypertension. Several experiments were done in which various doses (50 to 800 µg) of 6-HDA were injected intraventricularly. In one experiment (100 µg 6-HDA centrally) the animals developed significant increases in blood pressure, however, several subsequent experiments failed to confirm this finding. In all experiments, however, substantial (> 50%) decreases in brainstem NE were found. From these experiments one could not substantiate a clear inverse relationship between brainstem NE nor could the peripheral sympathetic system be implicated in the development of hypertension.

Catecholamine Repletion. Attempts were made to elevate the levels of catecholamines in the central nervous system of the SHR. Administration of the catecholamine precursor L-dopa (200 mg/kg) to SHR with or without the peripheral decarboxylase inhibitor MK-486 (L-hydrazino-α-methyldopa), 100 mg/kg, resulted in an acute and significant decrease in blood pressure. Continued administration of MK-486 and L-dopa for several days in the SHR continued to depress blood pressure. Chronic treatment of animals, however, did not appear to be feasible since the animals receiving this drug treatment ceased to grow normally. Using several different dosage schedules of dopa with or without a decarboxylase inhibitor and/or a monoamine oxidase inhibitor, a significant inverse correlation between blood pressure and brainstem NE was observed. Since treatment of animals with L-dopa causes a decreased serotonin content of the brainstem, the effect of the specific serotonin depletor p-chlorophenylalanine (PCPA) was studied. In normotensive Wistars and SHR a slight but significant increase in blood pressure occurred following a serotonin depleting dose of 300 mg/kg of PCPA. Administration of the normal serotonin precursor, 5-HTP, to normal or SHR did not affect blood pressure. It was therefore concluded that the ability of L-dopa to reduce blood pressure was perhaps related to catecholamine repletion, but probably not to serotonin depletion.

Significance: Hypertension in man is a complex disorder probably resulting from a variety of factors. The genetically hypertensive rats appear to be an excellent animal model of essential hypertension in man. The current work raises the possibility that one of the factors in the hypertension of these animals is a decrease in the number of or activity of noradrenergic neurons in the central nervous system. It is interesting to note that one of the side effects in patients receiving L-dopa for Parkinsonism is hypotension. At a metabolic level this work also raises the possibility that of the enzymes involved in the biosynthesis of NE from tyrosine, tyrosine hydroxylase may not be the rate limiting enzyme in all conditions. In other words the NE biosynthetic enzymes may be reasonably well balanced in vivo and a 50% decrease in aromatic L amino acid decarboxylase may be sufficient to affect the level of NE in tissues.

Proposed Course of Project: Studies are now in progress using ^{3}H-dopa and ^{14}C-tyrosine as precursors of NE to determine if the SHR have a lower NE synthetic rate and if in this instance the decarboxylase could be rate limiting in the central nervous system. Systems have been reestablished for the complete separation and measurement of tyrosine, dopa, dopamine and norepinephrine in tissues and experiments are being done to evaluate central and peripheral catecholamine metabolism in SHR.

Publications:

1. Yamori, Y., Lovenberg, W., and Sjoerdsma, A.: Norepinephrine metabolism in brainstem of spontaneously hypertensive rats. Science 170: 544-546, 1970.

Serial No. NHLI-100
1. Experimental Therapeutics Branch
2. Section on Biochemical Pharmacology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on a Pineal Gland Protein Kinase

Previous Serial Number: None

Principal Investigator: Joseph Fontana, Ph. D.

Other Investigators: Walter Lovenberg, Ph. D.

Project Description:

Objectives: It has been observed that norepinephrine stimulates the production of melatonin in the pineal gland. This stimulation appears to operate through a cyclic AMP mechanism. The objective of the present investigation is to probe for the existence of a cyclic-AMP sensitive control mechanism in pineal extracts, characterize it and determine its physiological significance.

Methods: Cyclic-AMP-dependent protein kinases which catalyze the phosphorylation of casein, protamine and/or histone by ATP have been found in muscle, liver, brain, rabbit, reticulocytes, adipose tissue and bacteria. Utilizing the hypothesis that such a cyclic-AMP dependent kinase also exists in the pineal gland, a protein kinase assay was adapted to the pineal system. The assay simply involves the phosphorylation by ATP-γP^{32} of either endogenous or exogenous (histone, casein, etc.) substrate in the absence or presence of cyclic AMP.

The sources of pineals employed in the study are Pel-Freeze and Swift and Co. Rat pineals are freshly obtained from Sprague-Dawley rats.

Major Findings: Cyclic-AMP dependent kinase activity was found in bovine pineal homogenates. The activity was partially purified by $(NH_4)_2SO_4$ precipitation (0-33%) and passage through DEAE-cellulose using step elution with 0.1 and 0.3M potassium phosphate pH=7.0 buffers. Two peaks of protein kinase activity were eluted from the DEAE-cellulose column-one eluting a 0.1M potassium phosphate and the second at 0.3M potassium phosphate. The entire activity from the latter peak was stimulated from 10-30 fold by cyclic-AMP. Maximum stimulation occurred at 5×10^{-6}M cyclic-AMP. Although a variety of proteins, including histone, phosvitin, casein and bovine serum albumin were able to act as phosphate acceptors for the enzyme, histone was far more effective in low amounts on a weight basis than were any of the other proteins studied. Lysine rich histone and histone seem to give similar results and both are better substrates than arginine rich histone.

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It has also been possible to detect protein-kinase activity in individual rat pineals (≈ 1 mg wet weight). Preliminary experiments show that this activity is slightly stimulated by cyclic-AMP. Conditions must be further developed to confirm this cyclic-AMP stimulation.

Significance and Proposed Course of Project: The diurnal variation of the enzyme levels in the melatonin pathway in pineals offers a unique means of examining one possible physiological significance of the cyclic-AMP dependent protein kinase activity. Further studies will examine the response of the cyclic-AMP dependent protein kinase to variations in environmental lighting and stimuli from the central nervous system and correlate these responses to variations in the levels of the melatonin biosynthetic enzymes and phosphorylation of endogenous histones. These studies may elucidate the role of histone phosphorylation. We will also attempt to purify and characterize the protein kinase(s) from the pineal.

Publications: None

Serial No. NHLI-101
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Naturally Occurring Vasoactive Substances

Previous Serial Number: NHLI-141

Principal Investigator: Ronald G. Geller, Ph.D.

Other Investigators: Wybren DeJong, M.D., Ph.D.
Renee Ray-Chung Chen, Ph.D.
Joseph Pierce, D.V.M.
Michael Beaven, Ph.D.
Takenori Tanimura, Ph.D.
John J. Pisano, Ph.D.

Cooperating Units: Laboratory of Animal Medicine and Surgery, NHLI

Project Description:

Objectives: To characterize the vasoactive substances 1) found in dog plasma following bilateral renal artery ligation, 2) in the venom of the bald-faced hornet, and 3) which are synthetic analogs of ranatensin, an undecapeptide isolated from frog skin.

Methods: Various isolated smooth muscle preparations and blood pressure recordings from experimental animals were employed.

Major Findings: A substance which raised blood pressure in the pentolinium treated rat was found in the plasma of dogs after bilateral renal artery ligation. It was isolated from the 40-70% ammonium sulfate fraction. It has been suggested that this pressor substance may be a protein, angiotensin I, angiotensin II, or a peptide bound to a protein. The nature of the blood pressure response in the rat and the lack of a response on isolated rat uterus or rat colon suggests that the substance is like angiotensin I, but is not angiotensin II. The pressor activity is abolished by boiling the plasma indicating the possibility of an angiotensin I-protein complex.

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The venom of the bald-faced hornet was found to contain a potent hypotensive agent on rat blood pressure. Experiments using the isolated rat uterus and guinea pig ileum indicated that the venom contained in addition serotonin (1-5 μg/sac) and and histamine (2.5 μg/sac). It did not contain acetylcholine or bradykinin. Histamine and serotonin were also measured using fluorescence assays. The hypotensive response in the rat was not completely blocked by antihistamine and anti-serotoninergic agents. This suggests that another substance possibly a peptide, is present, but has no action on the isolated preparations.

Ranatensin is an undecapeptide isolated from frog skin with the amino-acid sequence PYR-VAL-PRO-GLN-TRP-ALA-VAL-GLY-HIS-PHE-MET-NH_2. It has a characteristic spectrum of pharmacological activity which includes a potent depressor action on the blood pressure of monkeys and a potent stimulant action on the isolated rat uterus. The following analogs of ranatensin have been synthesized and their biologic activities studied. (See table).

Analogs which lowered blood pressure were also active on the uterus, but one, C-8, was much more active in lowering blood pressure than in contracting the uterus.

Significance to Biomedical Research and Institute Program: An analog of ranatensin which lowers blood pressure, but does not contract other smooth muscles could be a suitable one for testing in hypertension. One which acts on the uterus, but does not lower blood pressure would be of interest as an abortive agent. The isolation and characterization of the pressor peptide (or protein) associated with the initiation and maintenance of hypertension of renal origin could help clarify the role of the renin-angiotensin system in renal artery stenosis.

Proposed Course of Project: Other bioassay systems will be used to study the structure-activity relationships of ranatensin and its synthetic analogs. The vasoactive substances from dog plasma and hornet venom will be further characterized. Attempts to find other naturally occurring vasoactive substances in frog skin and insect venom will continue.

Honors and Awards: None

	1 2 3 4 5 6 7 8 9 10 11 Pyr Val Pro Gln Trp Ala Val Gly His Phe Met-MH_2	Activity (%) Monkey BP	 Rat U
ranatensin		100	100
Val^6, Ala^7 ranatensin	——————Val^6,Ala^7——————	1	1
C-7	5 ————————	0	1
C-8	4 ————————	30	1
C-9	3 ————————	100	100
C-10	2 ————————	100	100
P+7	4 PYR ————————	0	1
P+8	3 PYR ————————	100	100
P+9	2 PYR ————————	100	100

Publications:

Geller, R.G., Govier, W.C., Pisano, J.J., Tanimura, T., and Clineschmidt, B.V.: The action of ranatensin, a new polypeptide from amphibian skin, on the blood pressure of experimental animals. Brit. J. Pharmacol. 40: 605-616, 1970.

Clineschmidt, B.V., Geller, R.G., Govier, W.C., Pisano, J.J., and Tanimura, T.: Effects of ranatensin, a polypeptide from frog skin on isolated smooth muscle. Brit. J. Pharm. In press, 1971.

Serial No. NHLI-102
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Sensitive Assay for Esterase Activity Employing Radioactive Substrates: Application to Plasma Kallikrein

Previous Serial Number: NHLI-132

Principal Investigator: Vida H. Beaven, Ph.D.

Other Investigators: John J. Pisano, Ph.D.
Jack V. Pierce, Ph.D.
Ronald G. Geller, Ph.D.
Harry Margolius, M.D., Ph.D.

Cooperating Units: None

Project Description:

Objectives: A sensitive assay for esterase activity, which employed p-tosyl-L-arginine-^{3}H-methylester (^{3}H-TAMe) as substrate and measured the ^{3}H-methanol released upon hydrolysis, was developed in this laboratory (see Project Report NHLI-132, 1969-70). This assay was successfully applied to the measurement of human urinary kallikrein esterase activity (see Project Report-Margolius, 1970-71). The objective of the present work is the modification of the assay conditions for the measurement of plasma kallikrein esterase activity.

Method: The method of assay for esterase activity was described in the previous report; however, the following modifications were adapted. The standard incubation contained ^{3}H-TAMe, buffer, soybean trypsin inhibitor or buffer, and plasma or partially purified human plasma kallikrein (HPK). The unchanged ^{3}H-substrate was separated from the ^{3}H-methanol by extraction of the ^{3}H-methanol from the incubation mixture into a toluene-Liquifluor solution. The toluene solution was assayed for tritium. Kallikrein esterase was considered to be that esterase activity which was inhibited by soybean trypsin inhibitor.

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Major Findings: The optimum assay conditions for plasma kallikrein esterase were as follows: The HPK production of ^{3}H-methanol was linear with HPK concentration to 0.0004 EU/0.05 ml incubation (1 EU = amount of enzyme which hydrolyzed 1 µmole ester/min) and with time to 30 minutes. The HPK esterase activity had a pH optimum of 7.5 and temperature optimum of 25°C. The activation of plasma kallikrein esterase was compared in human and dog plasma using (1) acetone (20%, v/v, for 4 hr at 25°C) or (2) kaolin (5 mg/ml, for one min at 25°C). Human plasma kallikrein esterase was activated 1.5 times more with acetone than with kaolin; dog plasma kallikrein esterase was activated 3 times more with kaolin than with acetone. Maximum activation of dog plasma kallikrein esterase activity was inhibited 40% in 5 minutes and 75% in 30 minutes after the addition of kaolin to the plasma. The maximum activation of dog plasma kallikrein with kaolin was also seen in one minute when measured in the rat uterus bioassay. Preliminary experiments with kaolin activated dog plasma indicated that the plasma kallikrein esterase activity, from both arterial and renal venous samples, was not influenced by short-term infusions of angiotensin (0.5 µg/kg/min, i.v.) or norepinephrine (10 µg/kg/min, i.v.). The same results were observed in plasma kallikrein measured in the bioassay.

Significance to Biomedical Research and Institute Program: The development of a reliable, rapid and sensitive esterase assay which can be applied to the measurement of plasma kallikrein esterase activity is crucial to establishing the significance of this enzyme in physiological and pathological conditions.

Proposed Course: Further evaluation of the assay is planned to establish that the esterase activity that is measured in the presence of plasma is all attributable to plasma kallikrein esterase. For example, specific activators and inhibitors of plasma kallikrein esterase will be tested to establish that the esterase activity inhibited by soybean trypsin inhibitor is kallikrein esterase and no other. Should a lack of specificity be discovered, other radioactive ester or peptide substrates will be evaluated. The selection of substrate will be based upon the ability of antibody to plasma kallikrein to inhibit the hydrolysis of the substrate; the hydrolysis of TAMe by urine kallikrein esterase is not inhibited by antibody to urine kallikrein. After the plasma assay is established it will be used to study a variety of clinical conditions including hypertension (See Project Report Margolius) asthma, edema, shock, fever, inflammation, pain, and arthritides of various etiologies.

Honors and Awards: None

Publications:

Beaven, V.H., Pierce, J.V., and Pisano, J.J.: A sensitive isotopic procedure for the assay of esterase activity: Measurement of human urinary kallikrein. Clin. Chim. Acta. 32: 67-73, 1971.

Serial No. NHLI-103
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Enzymes Involved in the Activation of Human Plasma Kallikrein

Previous Serial Number: NHLI-133

Principle Investigator: Marion E. Webster, Ph.D.

Other Investigators: Vida H. Beaven, Ph.D.
Yumiko Nagai, Ph.D.
Jack V. Pierce, Ph.D.
John J. Pisano, Ph.D.

Cooperating Units: None

Project Description:

Objectives: The kallikrein which is present in human plasma is normally found as an inactive precursor called prekallikrein. The mechanism by which this enzyme is activated in human blood is not fully understood. Nothing is known of the chemical bonds cleaved during the conversion of prekallikrein to kallikrein although it is assumed to be mediated by an enzyme. This inactive enzyme precursor is believed to be activated by other enzymes, and it is currently proposed (Webster, Fed. Proc. 27, 84, 1968) that a series of pre-enzyme to enzyme conversions results in the activation of plasma kallikrein which acts on the final substrate, kininogen, to release bradykinin. Two of the enzymes believed to be involved in the activation of prekallikrein are Hageman factor and pF/dil. This study is an investigation of the enzymes involved in the activation of prekallikrein. Attempts are being made to prepare both the active form and inactive precursor of the different enzymes and to arrange them in an orderly sequence.

Methods: Plasma kallikrein activity was determined by measuring the generation of kinins using the direct bioassay on the isolated guinea pig ileum with heated (60° for 1/2 hour) plasma as substrate.

When intact plasma is used as substrate in this bioassay, the activity due to both plasma kallikrein and the activator of prekallikrein are determined. TAMe esterase activity was determined using ^{3}H-TAMe as previously described (Beaven, NHLI Project Report No. 132, 1970). Hageman factor and PTA activity are performed in the usual manner using human and bovine plasma deficient in these factors. Both inactive and active factors are estimated in the presence of kaolin whereas in the absence of kaolin only the active factor is determined.

Major Findings: Previous results had shown that chromatography of acetone activated human plasma on DEAE-cellulose resulted in the separation of a number of peaks (II, III, IV, and V) which would generate kinin activity from both heated and non-heated plasma suggesting that they were activators of prekallikrein. Attempts to arrange these enzymes in an orderly sequence by kinetic studies or by differentiating them by inhibitors was unsuccessful, although they could readily be differentiated from plasma kallikrein, (peak I). Active Hageman factor and/or active PTA were also present in the fractions from this column. The relative activity of the clotting factors was similar in the various fractions, but varied from that shown by the activators of prekallikrein. Peak V, which in some columns represented the major portion of the activator activity, was further purified by chromatography on hydroxyappatite. A single sharp peak of activity was obtained and the enzyme had been purified 2-6,000-fold when compared to the starting plasma with an over-all yield of 36%. These fractions still retained TAMe esterase activity, although only a small portion of that found in Peak V. Repeated freezing and thawing of these fractions resulted in loss of activity, possibly due to the low concentration of protein in the fractions. Peaks II and III were rechromatographed on DEAE-cellulose to investigate their possible conversion to peak V as found by other investigators (Kaplan and Austen, J. Immunology 105, 802, 1970). However, in our hands, only a small portion of the activity (30%) rechromatographed in a position which might be considered equivalent to peak V. The remaining activity remained associated with the major protein peak and could now be clearly differentiated from the major TAMe esterase peak found in these fractions.

Since the recovery of plasma kallikrein and of the clotting factors from the DEAE-cellulose columns was poor (30% or less), alternate methods for the purification of these active enzymes were investigated. Plasma kallikrein can be recovered 600-fold purified with a 50% yield by adsorption and elution from a soy

bean trypsin inhibitor-sepharose (STI-sepharose) column NHLI Project Report-Pierce). The activators of prekallikrein, on the other hand, are found in the filtrates from these columns and can be purified 300-fold by adsorption and elution on a lima bean trypsin inhibitor-sepharose (LTI-sepharose) column. The observation that all of the activator activity can be removed by a single inhibitor column, together with the earlier inability to differentiate peaks II, III, IV and V by kinetic measurements or by inhibitors, suggests that multiple-forms of the same enzyme may be formed during acetone activation of human plasma. This view was strengthened when it was found that treatment of the STI-sepharose filtrates with urea, guanidine or potassium thiocyanate resulted in a two-fold increase in biological activity.

The above procedures, however, cause a loss in the clotting factors. For example, in one such preparation only 38 and 62% of the active Hageman and PTA activities were recovered in the STI-sepharose filtrates and only 2.6 and 10% respectively in the final LTI-sepharose filtrates. The method of Speer et al. (Thromb. Diath. Haem. 14, 1-11, 1965) for the preparation of active Hageman factor was first investigated. By this procedure the Hageman factor is adsorbed and eluted from deactivated supercel and further purified by isoelectric precipitation, ammonium sulfate precipitation and chromatography on CM-Sephadex. Following this procedure active Hageman factor was obtained from the supercel eluates. However, adjustment of the pH to 5.2 for the isoelectric precipitation caused losses in activity and no active precipitate formed. Ammonium sulfate precipitation of the supercel eluates resulted in the recovery of most of the active Hageman factor activity while achieving a 300-fold purification. Attempts to further purify by CM-Sephadex chromatography as described by these authors were unsuccessful and all activity was lost. Also, it was found that the major portion of active Hageman factor (PTA activity was not measured) could be removed from acetone activated human plasma by adsorption on deactivated supercel, leaving most of the prekallikrein activator and plasma kallikrein in the filtrate. Since acetone activated plasma appears to contain all of the Hageman factor activity of the starting plasma, this separation of active Hageman factor and prekallikrein activator makes it unlikely that the activator is derived from active Hageman factor as proposed by others (Kaplan and Austen, J. Immunol. 105, 803, 1970; Wuepper et al., J. Immunol. 105, 1307, 1970).

In addition to isolating the active components of this enzyme system as described above, preliminary attempts have been made to

isolate the inactive components. Plasma deficient in Hageman factor contains prekallikrein (Webster and Ratnoff, Nature 192, 180, 1961), but does not form active enzyme on contact with negatively charged surfaces or on treatment with acetone. Chromatography of this plasma on DEAE-cellulose gave a partially purified prekallikrein preparation, the activity being associated only with those proteins which did not bind to DEAE-cellulose. The activity of these fractions was determined by measuring the increase in esterase activity generated by the prekallikrein activator. Preliminary experiments indicated that the pH of the activation step could be varied from 7.0 to 8.5 with no change in activity. The amount of esterase activity generated at room temperature by this pre-incubation increased from three to ten minutes and remained constant or decreased slightly if the incubation was prolonged for periods of time up to 30 minutes. With the concentration of enzyme chosen in these studies a linear relationship between concentration of substrate and generation of esterase activity was obtained using a five-minute incubation period of pH 8.5. The fractions from this column also generated esterase activity when trypsin, rather than the activator of prekallikrein, was used as the enzyme. Trypsin, however, was not as specific as the activator of prekallikrein since it also generated large amounts of esterase activity from another unidentified pre-enzyme which adsorbed to the DEAE-cellulose.

Attempts were also made to find an inactive precursor of the activator of prekallikrein. In these experiments, the fractions from the DEAE-cellulose columns of Hageman deficient plasma and of acetone activated Hageman deficient plasma were incubated with partially purified active Hageman factor and with a source of prekallikrein. No increased generation of esterase activity was found. However, duplicate determinations were poor as were attempts to repeat the results on subsequent days. It was found that part of the difficulty was due to the extreme lability of the prekallikrein fractions which can only be frozen and thawed once and the remaining difficulties due to the large amount of protein adsorbed to the IRC-50 resin. These observations led to the development of the extraction method for the determination of human plasma kallikrein (Beaven, NHLI Project Report No.).

The preparation of inactive Hageman factor was also attempted. Initially, plasma containing 40 μg/ml hexadimethrine bromide was adsorbed with deactivated supercel and eluted in a manner similar to that described for the preparation of active Hageman factor. The eluates from these adsorptions contained much higher Hageman

factor activity (4-5 fold) than those eluates prepared from plasma in the absence of hexadimethrine bromide. They also contained a 4-fold increase in content of activator of prekallikrein, while the kallikrein content of the two eluates varied by less than two-fold. Since the supernatant from these adsorptions were essentially devoid of Hageman factor activity, this suggested that the presence of hexadimethrine bromide was either directly preventing the inactivation of Hageman factor and/or prekallikrein activator or was delaying the activation of the Hageman factor on the supercel and thus preventing its inactivation by the plasma proteins in the filtrate. Hexadimethrine bromide at this concentration did not prevent the activation of Hageman factor since only 50% or less of the Hageman factor was estimated to be in its inactive form. Also when these eluates were fractionated with ammonium sulfate in the presence of hexadimethrine bromide they appeared to become fully activated, giving a two-fold increase in both active Hageman factor and prekallikrein activator. The parallelism is these fractions between the active Hageman factor activity and the content of prekallikrein activator was striking suggesting that both were derived from the same molecule (inactive Hageman factor). In order to further substantiate this hypothesis, efforts are currently being made to prepare inactive Hageman factor essentially devoid of active Hageman factor. Preliminary results have provided such a preparation but require confirmation.

In summary, progress is being made in isolating and characterizing both the active and inactive forms of the various enzymes involved in the activation of plasma prekallikrein. No conclusive evidence is yet available that more than one activator is involved. In fact the current evidence would suggest that multiple forms of the same activator are generated during acetone activation of plasma. Also, the present evidence does not favor the view that the prekallikrein activator is formed from active Hageman factor as proposed by others, but rather suggests that inactive Hageman factor forms at least two active products, the activator of prekallikrein (pF/dil?) and active Hageman factor.

Significance to Biomedical Research and Institute Program: The generation of kinins by plasma kallikrein has been implicated in a number of pathological conditions such as hereditary angioneurotic edema, gouty and rheumatic arthritis, asthma, pulmonary edema, reactions to blood transfusions, infection by pathogenic organism, pancreatitis, etc. A complete understanding of this system together with isolation and characterization of the various

enzymes, could lead to the development of inhibitors which would be therapeutically useful.

Proposed Course of Project: Continued investigation of the enzymes involved in the activation of prekallikrein. Methods will be developed for the further purification of both the active form and inactive precursor of the different enzymes and an attempt will be made to arrange them in an orderly sequence.

Honors and Awards: None

Publications:

Webster, M.E., Pierce, J.V., and Sampaio, M.U.: Studies on antibody to bradykinin. Adv. Exp. Med. Biol. 8: 57-64, 1970.

Webster, M.E.: Recommendation for nomenclature and units. In Erdos. E.G. (Ed), "Bradykinin, kallidin and kallikrein". Handbook of Experimental Pharmacology, 25: 659-665, 1970.

Webster, M.E.: Kallikreins in glandular tissues, In Erdos, E.G. (Ed), "Bradykinin, kallidin and kallikrein", Handbook of Experimental Pharmacology, 25: 131-155, 1970.

Webster, M.E., and Prado, E.S.: Glandular kallikreins from horse and human urine and from hog pancreas. In Lorand, L. and Perlman, E.G. (Eds), Methods in Enzymology 19: 681-699, 1970.

Goodfriend, T.L., Webster, M.E., and McGuire, J.S.: Complex effects of antibodies to polypeptide hormones. J. Clin. Endocrinol. Metab. 30: 565-572, 1970.

Skinner, S.N., Jr., and Webster, M.E.: Acetylcholine and functional vasodilatation in the submaxillary gland of the cat. J. Europ. Pharmacol. 12: 271-275, 1970.

Serial No. NHLI-104
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Analysis of Amino Acid Phenylthiohydantoin by Gas Chromatography

Previous Serial Number: NHI-105

Principal Investigator: John J. Pisano, Ph.D.

Cooperating Units: None

Project Description:

Objectives: The analysis of amino acid phenylthiohydantoin derivatives (PTH) is a major problem in the determination of the amino acid sequences of polypeptides and proteins. Current methods of analysis involve thin layer and paper chromatographic techniques which are inadequate. The present work was undertaken to improve the analysis of amino acid PTHs and in particular to explore the potential of gas chromatography, a technique noted for its high speed of analysis, resolving power, sensitivity, and ease of quantitation.

Major Findings: Advances in the gas chromatography procedure for the identification of amino acid thriohydantoins include: 1) a new blend of silicone stationary phases (7.33% SP-400, 5.33% OV-210, and 0.66% OV-225) which provides superior resolving power than higherto obtained with single phases or earlier blends; 2) the use of helium as carrier gas which is superior to the more commonly used nitrogen andargon, in that it gives the best resolution and the greatest range of flow rates with no sacrifice in efficiency; and 3) high temperature conditioning which gives more efficient columns with significantly less bleed (baseline rise) during temperature programming. The methylthiohydantoins were also examined. All derivatives, except the seryl (unstable) and arginyl (non-volatile) were separable on a column using the stationary phase, OV-225.

Automated sample injection has been evaluated and its feasibility demonstrated with standard PTH derivatives obtained for the automated degradation currently under study. The latter samples contain impurities which may hasten the destruction of the derivatives in the sample holder.

A new solvent, dioxane, containing the antioxidant diethyldithiocarbamate is superior to all other solvents tested for preparing standard solutions of the amino acid PTHs. Unlike any previous solvent tested (or mixture of solvents) it dissolves all the derivatives. An added advantage (due to the antioxidant) is the greater stability of the derivatives in this solvent.

A comparison has been made between the gas chromatographic and a new mass spectrometric method developed by Dr. Henry Fales, Laboratory of Chemistry, NHLI. The latter technique employs chemical ionization mass spectrometry. Sperm whale myoglobin was degraded by Dr. Bryan Brewer, Molecular Diseases Branch, NHLI, using the protein sequenator. It was shown that the gas chromatographic method is much simpler to employ, but the MS method has the potential of greater sensitivity and speed of analysis.

Significance to Biomedical Research and Institute Program: The technique greatly facilitates the analysis of amino acid PTH derivatives obtained in the sequential degradation of polypeptides and proteins. Included in these classes of substances are hormones, antibodies, enzymes, structural proteins, peptides, and other substances of biomedical importance. The structural determination of these important substances has been greatly hampered by analytical difficulties. With automation of the Edman degradation by Edman and the now commercially available protein sequenators, the gas chromatographic procedure for the analysis of the amino acid PTH derivatives (Edman derivatives) is even more attractive because of the speed, sensitivity, resolving power and ease of quantitation of the technique.

Proposed Course of Project: Extension of the automation of the gas chromatographic procedure developed with standards to PTH derivatives obtained from the protein sequenator. Development of a protocal which would allow identification of a PTH amino acid in less than 50 minutes. Development of a gas chromatographic procedure for the analysis of pyroglutamic acid, N-acetyl and N-formal amino acids. Assists other laboratories at NIH in setting up the procedure. Collaborate with other laboratories in polypeptide sequence analysis.

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Honors and Awards: None

Publications:

Pisano, J.J., Bronzert, T.J., and Brewer, H.B., Jr.: Advances in the gas chromatographic analysis of amino acid phenyl- and methylthiohydantoins. Anal. Biochem. In press.

Fales, H.M., Nagai, Y., Milne, G.W.A., Brewer, H.B., Jr., Bronzert, T.J., and Pisano, J.J.: The use of chemical ionization mass spectrometry in the analysis of the amino acid phenylthiohydantoin derivatives formed during the Edman degradation of proteins. Anal. Biochem. In press.

Serial No. NHLI-105
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Application of Countercurrent Chromatography to the Isolation and Characterization of Substances of Biochemical Interests

Previous Serial Number: NHLI-136

Principle Investigator: Hisanobu Yoshida, Ph.D.

Other Investigators: John J. Pisano, Ph.D.
Yoichiro Ito, M.D.
Robert L. Bowman, M.D.

Cooperating Units: Laboratory of Technical Development

Project Description:

Objectives: To perfect and apply the technique of countercurrent chromatography to the solution of problems in the isolation and characterization of compounds of biomedical interests.

Methods: Design and construction of suitable equipment. Development of theory and practice of countercurrent chromatography.

Major Findings: Droplet countercurrent chromatography (DCCC), a new chromatographic technique developed in our laboratory, was applied to the separation of milligram quantities of 2, 4 dinitrophenyl derivatives of amino acid (NHLI-136) with an efficiency comparable to gas chromatography. The promise of this method is being fulfilled with its application to the analysis of new classes of compounds and with the construction of an even simpler unit consisting only of teflon tubing. This unit has been used in the separation of the following model peptides: bradykinin, kallidin, and angiotensin; ranatensin from crude methanolic extracts of frog skin and insulin from its A and B chains. Solvent systems used are similar to those employed in countercurrent distribution and include sec-butanol-trifluoracetic acid- water, n-butanol-acetic acid-water, sec-butanol-1% dichloreacetic acid

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and n-butanol-0.1% acetic acid-pyridine. Reproducibility of the method is excellent. It has also been shown that the elution volume may be calculated knowing the partition coefficient and the volume of stationary phase. Recovery of the sample is quantitative with as little as 1 μg of material.

Another form of countercurrent chromatography, gyration locular countercurrent chromatography (developed by Drs. Ito and Bowman), is being refined and evaluated. Several kinds of locular columns (all teflon, teflon-glass, glass-lined thru hole in the teflon spacers, etc.) have been constructed and applied to the separation of bradykinin, kallidin, and angiotensin and Val-t-RNA or Phe-t-RNA from a mixture of amino acyl-t-RNAs. In general the method has higher resolving power and speed of analysis than droplet countercurrent chromatography. However, it is more difficult to execute and has a lower capacity.

Significance to Biomedical Research and Institute Problems: Standard ion exchange, and liquid-liquid chromatographic procedures are often not satisfactory for the separation of neutral, organic-soluble compounds of biological interest. The classic countercurrent distribution technique is often used, but it is cumbersome. Liquid-liquid chromatographic methods employing solid supports often exhibit tailing due to adsorption of the solutes to the support, and the capacity of this technique is low.

Countercurrent chromatography on the other hand is an all-liquid separation technique ideally suited for any substance which partitions between two immiscible solvents and is free of adsorptive effects. The latter is most important in the separation of peptides and many other biological substances which adsorb (often irreversibly) to the solid supports used in conventional chromatography.

Proposed Course of Project: Having established the usefulness of countercurrent chromatography in the separation of peptides and ribonucleic acids, the technique will be extended to other classes of substance such as lipids, pesticides, carbohydrates, etc. Practical applications are anticipated in the isolation and identification of naturally occurring peptides and peptides obtained in the degradation of proteins undergoing amino acid sequence analysis.

Honors and Awards: None

Publications:

Tanimura, T., Pisano, J.J., Ito, Y., and Bowman, R.L.: Droplet countercurrent chromatography, Science, 169: 54-56, 1970.

Serial No. NHLI-106
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Structure of Villikinin

Previous Serial Number: NHLI-143

Principal Investigator: John J. Pisano, Ph.D.

Other Investigators: Eszter Kokas, Ph.D.

Cooperating Units: Physiology Department
University of North Carolina
Medical School

Project Description:

Objectives: To isolate and determine the structure of villikinin, a substance obtained from intestinal mucosal extract which has a specific stimulant action on intestinal villous motility.

Methods: Crude intestinal mucosal extract is desalted on Dowex-50 further purified by gel filtration on Sephadex G-25 or Bio-Gel P-4 and finally chromatographed on Beckman UR-30 resin using a pyridine acetate buffer or on SE Sephadex using an ammonium acetate gradient. Material is assayed in vivo by measuring its activity on pumping action of exposed intestinal villi of dogs.

Major Findings: Although the previously reported procedure for obtaining an active villikinin fraction from crude mucosal extract resulted in over a 100-fold purification, the peaks obtained from both gel filtration on Sephadex G-25 and cation exchange chromatography in a pyridine acetate buffer have been unsatisfactorily broad. Bio-gel P-4 is currently under evaluation as a substitute for Sephadex G-25. A well-defined peak of activity with an apparent molecular weight between 2000 and 4000 was obtained on a Bio-gel P-4 column using bovine mucosal extract. Canine mucosal extract on Sephadex G-25 gave a broad peak with an apparent molecular weight of about 1000. At this time it is impossible to judge whether the apparent disparity is due to a real difference

between canine and bovine villikinin, or is a result of differing experimental procedures. Canine extract must be tested on Bio-gel P-4.

When either active product obtained by gel filtration is chromatographed in a pyridine acetate buffer on cation exchange resin, it is strongly retarded and elutes slowly in a very broad peak. Cation exchange chromatography using SE-Sephadex is now being studied as a purification step to follow gel filtration. Bovine mucosal extract which had been processed through the Dowex-50 desalting step and Bio-gel P-4 chromatography was tested with several enzymes. Like canine material it was inactivated by Pronase, papain and chymotrypsin and not inactivated by trypsin. These results are significant because they indicate that we are purifying a specific biologically active substance in as much as the partially purified material behaves identically to the crude extract treated with the above enzymes. What is more, finding the same material in canine and bovine intestinal extracts suggests its widespread occurrence and physiological importance.

Significance to Biomedical Research and Institute Program: Villikinin may be a specific hormone which controls intestinal villous motility. The theory can only be proven when the substance is isolated and its structure determined.

Proposed Course of Project: With the discovery of villikinin in bovine intestinal extracts abundant supplies of material is assured. Various purification schemes will be tested. When pure material is obtained its amino acid sequence will be determined and confirmed by synthesis. Pharmacological studies will be also undertaken to determine the physiological importance of villikinin in man.

Honors and Awards: None

Publications: None

Serial No. NHLI-107
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Unusual Linkages in Peptides

Previous Serial Number: NHLI-140

Principal Investigator: John J. Pisano, Ph.D.

Other Investigators: John S. Finlayson, Ph.D.
Yumiko Nagai, Ph.D.

Cooperating Units: Division of Biological Standards
Laboratory of Blood and Blood Products

Project Description:

Objectives: The objectives in this study were to determine the biochemical lesion in Factor XIII deficiency, to measure the degree of crosslinking in normal human plasma and in plasma from patients with Factor XIII deficiency, and to determine the extent of the α and γ chain involvement in crosslinking, for example, the number of crosslinks contributed by each chain.

Methods: A technique has been developed for clotting fibrin directly in human plasma and removing the clot by winding it on a stirring rod as it forms so that the clots can be washed, solubilized and carried through previously described procedures for measuring ε-(γ-glutamyl)lysine crosslinks. Both an enzymic digestion method for measuring crosslinks directly and a chemical procedure for measuring crosslinked lysine by cyanoethylation and subsequent acid hydrolysis are used. Qualitative determination of the extent of crosslinking was determined by disc gel electrophoresis.

Major Findings: Fibrin clots which were formed with normal plasma contained approximately 6 moles ε-(γ-glutamyl)lysine/mole fibrin, whereas those formed in plasma from individuals with Factor XIII deficiency contained little or none of this crosslink (0.02-0.64 moles/mole fibrin). Partial supplementation of the deficient plasma with Factor XIII commensurately increased the number of crosslinks.

To determine the extent that each of the chains of fibrin participated in normal crosslinking, polymerized fibrin was reduced in the presence of denaturing agents. γ-Chains were recovered as dimers which contain a maximum of two crosslinks/dimer. α-Chains were recovered as polymers. Examination of high molecular weight and intermediate molecular weight α-polymers revealed 4.8 and 3.2 crosslinks/2 α chains, respectively.

Significance: The fact that little or no crosslink formation occurred in the plasma of Factor XIII-deficient patients furnishes an explanation and biochemical basis for the laboratory findings usually associated with this deficiency (viz., friable clots, solubility of clots in 5 M urea, 1% monochloroacetic acid). Although it is not certain that the inability to form ε-(γ-glutamyl) lysine is the sole molecular defect in Factor XIII deficiency, the importance of the crosslink in hemostasis appears established.

The finding of 6 crosslinks per mole for normal fibrin has shed new light on the nature of fibrin crosslinking. The α-chains are more highly crosslinked than γ-chains and contribute an average of 4 crosslinks per mole.

Proposed Course of Project: This project will be continued to determine the exact extent of involvement of the α-chains in fibrin crosslinking, to determine the amount of normal plasma or purified factor needed to supplement Factor XIII-deficient plasma to form the normal 6 crosslinks/mole, to examine fibrin crosslinking in clots formed *in vivo*, and to examine other proteins for the ε-(γ-glutamyl)lysine crosslink.

Honors and Awards: None

Publications:

Pisano, J.J., Finlayson, J.S., Peyton, M.P., and Nagai, Y.: ε-(γ-Glutamyl)lysine in fibrin: lack of crosslink formation in factor XIII deficiency. *Proc. Nat. Acad. Sci.* 68: 4, (1971) in press.

Serial No. NHLI-108
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Preparation of Affinity Adsorbents

Previous Serial Number: NHLI-137

Principal Investigator: Jack V. Pierce, Ph.D.

Other Investigators: Larry J. Loeffler, Ph.D.

Cooperating Units: None

Project Description:

Objectives: To prepare chemically reactive derivatives of agarose, glass beads, or other materials useful in affinity chromatography, which can be coupled under mild conditions with a wide variety of proteins, peptides, and inhibitors of interest to provide useful affinity adsorbents for the purification of materials of biological interest. Initially, to prepare an insoluble trypsin adsorbent of sufficient binding capacity for practical use in the isolation and purification of trypsin inhibitors from potatoes, peanuts, and other sources. To bind these inhibitors themselves to insoluble supports for possible use in purifying enzymes of the plasma kallikrein system.

Method: Covalent binding of trypsin to Sepharose or porous glass beads, using strategies which will result in the trypsin molecule being at a sufficient distance from the support backbone to allow full activity in binding inhibitors.

Major Findings: Trypsin insolubilized by direct coupling to cyanogen bromide-activated Sepharose 4B was found to have less than 50% of the theoretical activity in binding such inhibitors as soybean trypsin inhibitor and lima bean trypsin inhibitor, probably due to the proximity of the trypsin molecule to the support backbone.

A high capacity trypsin column has now been prepared, employing Sepharose 4B as the support, but interposing a chain of about 16 atoms between the trypsin and the support. The methyl ester of 11-aminoundecanoic acid was prepared by esterification using thionyl chloride in methanol. The product was coupled to cyanogen bromide-activated Sepharose 4B, affording an insoluble ester derivative containing 0.30 m equivalents of ester groups per 100 g of Sepharose (original wet weight). Treatment of this material with hydrazine hydrate in methanol gave a hydrazide derivative. Treatment of the hydrazide in dilute HCl with $NaNO_2$ gave the azide, which was reacted with trypsin in buffer at pH 8-9. Under the most favorable conditions, an insoluble trypsin was obtained which contained about 7.1 mg of trypsin bound per g of Sepharose and which bound 3.2 mg of soybean trypsin inhibitor per g of Sepharose (about 70% of theory).

A similar series of reactions was carried out using glycine methyl ester and afforded an insoluble trypsin with a considerably lower capacity for soybean trypsin inhibitor.

A trypsin-porous glass derivative, prepared by a literature procedure, was found to be totally inactive in binding soybean trypsin inhibitor, although a large amount of trypsin had been bound.

Significance and Proposed Course of Project: The Sepharose-trypsin column discussed above promises to be of considerable utility for the large scale isolation of various trypsin inhibitors, some of which may also inhibit enzymes of the kallikrein system. Such inhibitors would be coupled to an insoluble support, perhaps using the Sepharose-azide intermediate mentioned above, to yield adsorbents for isolating the inhibitable enzymes. The Sepharose-azide intermediate may also prove useful for preparing a lysyl-bradykinin derivative for use in the purification of antibradykinin, which then in turn may be coupled to serve as the basis for a kinin radioimmuno-assay and to be used in the direct isolation of plasma kininogens terminating in the kinin moiety. Further developmental work on the chemistry of potentially useful coupling reactions on Sepharose, porous glass, and other suitable materials is planned.

Honors and Awards: None

Publications: None

Serial No. NHLI-109
1. Experimental Therapeutics Branch
2. Physiological Chemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemistry of the Kallikrein-kininogen-kinin System

Previous Serial Number: NHLI-134

Principal Investigator: Jack V. Pierce, Ph.D.

Other Investigators: Kjell Nustad, M.D. (Visiting Scientist)
Marion E. Webster, Ph.D.
Vida H. Beaven, Ph.D.
Ronald Geller, Ph.D.
Renne Chen, Ph.D.

Cooperating Units: Ungulate Unit, Animal Center Section, Laboratory Aids Branch, Division of Research Services (L. Stuart)

Project Description:

Objectives: Purification of human urinary and plasma kallikreins, Pf/dil, and Hageman Factor for purposes of characterization and of production of specific antiserums. Preparation from specific antibodies of affinity adsorbents for isolating the enzymes and possibly the proenzymes directly and specifically from human plasma and other fluids, and for devising specific biochemical and radioimmunochemical assays. Application of these assays to studies of human circulatory disease states, such as hypertention.

Purification of high molecular weight (HMW) and low molecular weight (LMW) kininogens from human and other mammalian plasma by means of affinity chromatography. Characterization of the isolated proteins. Isolation of piece B from kininogen II for physicochemical and biological studies. Preparation of an affinity adsorbent from piece B to isolate specific antibodies either from antiserums to kininogen II or from antiserums prepared to B.

Methods: Affinity chromatography on inhibitor-Sepharose columns of activated human plasma to obtain human plasma kallikrein and

Pf/dil; and on kininogen antibody-Sepharose columns of heated human plasma to obtain human plasma kininogens. Devising elution schemes for recovering the adsorbed proteins in biologically active form.

Major Findings: 1) Urinary Kallikreins. Human. Highly purified kallikrein from Type B urine was obtained by pressure dialysis, hydroxyapatite chromatography, and isoelectric focusing. The last step gave five TAMe esterase and biologically active peaks which were indistinguishable by disc gel electrophoresis: each gave three bands with the same mobilities. The bands from unstained disc gels of the main electrofocusing peak (HUK-B_3) were cut out, macerated, suspended in Freund's complete adjuvant, and injected intramuscularly into a sheep. Precipitating antibody was obtained after two booster injections. The antiserum inhibited the kinin-releasing ability of HUK, whereas normal sheep serum was not inhibitory. Since normal sheep serum inhibits the biological activity of HPK (human plasma kallikrein), antibody to HUK must be purified before it can be tested on HPK. A specific precipitate of HUK and antibody to HUK has been prepared as the first step in the isolation of pure, specific antibody.

Rat. Fractionation of a large lot of rat urine by methods described in our previous report gave two main TAMe esterase peaks, A and B; only B had kallikrein activity. Electrofocusing of peak B after hydroxyapatite chromatography gave four peaks of TAMe esterase and biological activity. Peaks B_1 and B_3 showed single disc gel bands, whereas B_2 and B_4 showed two bands: the lighter bands of B_2 and B_4 had the same mobilities as the B_1 and B_3 bands, respectively. All the bands were localized in a narrow mobility range. Molecular weight determinations of these RUK isoenzymes were obtained by SDS polyacrylamide disc gel electrophoresis, using ovalbumin and chymotrypsinogen A as markers. Bio-Gel P-200 gel filtration of B_3 showed coincident TAMe esterase activity and A_{280} peaks and no indication of impurities.

Antibody was elicited in a sheep by the disc gel method on RUK-B_3. A specific precipitate was prepared by mixing antiserum with pressure-dialyzed rat urine at equivalence. Separation of antibody and antigen was achieved by Sephadex G-100 gel filtration on 8 M urea. The enzyme was recovered free of antibody in 30% yield with a specific activity of 20 (μmole TAMe/min/A_{280} unit). The A_{280} peak appearing shortly after the column void volume contained only antibody. Another 14% of the starting enzyme activity with a specific activity of 10 was found in the region

between the two A_{280} peaks. Disc gel electrophoresis showed two sharp bands in the γ-globulin region for the antibody peak, three bands for the enzyme peak with the same mobilities found for a mixture of B_1 through B_4, and the same three bands plus a broad band with a mobility intermediate between the enzyme and the γ-globulin bands. The intermediate band had TAMe esterase activity, so was probably an antigen-antibody complex. The purified antibody was covalently bound to Sepharose by means of cyanogen bromide: the derivative was highly active in binding not only rat urinary kallikreins, but also the kallikreins in extracts of rat submandibular gland and pancreas.

Rat Urinary Kallikrein	Specific Activity	Recovery of Kallikrein Activity %	Molecular Weight SDS Gel	Molecular Weight Gel Filtration
B_1	14.5	2.2	36,200	
B_2	20.4	9.5	34,000	
B_3	22.8	20.2	33,400	38,500
B_4	18.8	9.4	32,600	
From Immune Precipitate				
Enzyme Frac. 1	20.0	31		
Enzyme Frac. 2	10.4	14		

2) Human Plasma Kallikrein. Small amounts of this enzyme have been isolated by affinity chromatography of acetone-activated human plasma on STI (soybean trypsin inhibitor)-Sepharose columns. After washing the column with suitable solvents, about 25% of the starting HPK TAMe esterase and biological activities were recovered in the 5 M guanidine hydrochloride eluate. The degree of purity of such preparations has not yet been tested.

3) Human Pf/dil. This enzyme is thought to effect the activation of plasma kallikrein and is itself present in fresh human plasma in an inactive form. It appears to be derived from Hageman factor. The Pf/dil activity in acetone-activated plasma is not bound to STI-Sepharose, being quantitatively recovered in the filtrate, but can be weakly bound to an LTI (lima bean trypsin inhibitor)-Sepharose column in the presence of 0.15 M NaCl. A purification of between 200- and 400-fold in nearly quantitative

yield has been achieved on a small scale. The Pf/dil activity of the STI-Sepharose column filtrate can be doubled by treatment with the chaotropic solvents 8 M urea, 5 M guanidine hydrochloride, and 4 M sodium thiocyanate. After dialysis against saline, then 0.01 M Tris Cl, pH 7.5, the Pf/dil activity behaves the same on LTI-Sepharose columns as the untreated material.

4) Mammalian Plasma Kininogens. Human. Human plasma collected under silicone conditions can be heated at 60° for 0.5 hour without apparent effect on either the HMW or LMW kininogens, whereas the enzymes of the kinin-generating system are destroyed. The kininogens present in such heated plasma have been adsorbed to antikininogen-Sepharose, either on columns or in batch operation. Elution with urea, sodium thiocyanate, or guanidine hydrochloride gave fractions containing both HMW and LMW kininogens in about 10% yield. Disc gel electrophoresis showed the presence of contaminants with the mobility of γ-globulins; whether these are specific antibody from the Sepharose column or plasma proteins co-adsorbed with the kininogens has not yet been determined.

Bovine. Sheep entiserum has been prepared from highly purified bovine LMW kininogen (supplied by E. Habermann) by the disc gel method. The specific precipitate formed at equivalence by mixing antiserum and bovine serum has been dissolved in 8 M urea and is being chromatographed on hydroxyapatite to separate the antibody from the antigen. As found earlier for the human kininogen-antibody precipitate, the antibody is not adsorbed and appears in the column filtrate free of antigen; antigen could be subsequently eluted free of antibody. Ouchterlony double diffusion plates can be used to follow the fractionation directly. An 8 M urea solution of antigen-antibody precipitate gives a precipitin ring, whereas an 8 M urea solution of only antigen (or antibody) gives a sharp precipitin line with antibody (or antigen); if both are present, but one is in excess of equivalence, a precipitin line and a partial ring are found.

Significance to Biomedical Research and Institute Program: The purification of the components of the plasma kallikrein system is crucial to investigations of its physiological function(s). This system is activated simultaneously with the blood coagulation system: Factor XII (Hageman Factor) of the latter appears to be an integral part of the former. There are a number of observations implicating the kallikrein system in inflammation, pain, immune reactions, the carcinoid syndrome, arthritides of various etiologies, and hereditary angioneurotic edema.

Proposed Course of Project: Specific antibodies to human urinary kallikrein will be isolated from the immune precipitate, bound to Sepharose for isolation of the enzyme directly from pressure-dialyzed urine and possibly of other glandular kallikreins, and tested for inhibition and binding of plasma kallikrein. Further purification of human plasma kallikrein, Pf/dil, and Hageman Factor will be performed for purposes of characterization and of production of specific antibodies for making affinity adsorbents.

Honors and Awards: None

Publications:

Webster, M.E., Pierce, J.V., and Sampaio, M.U.: Studies on antibody to bradykinin. Bradykinin and Related Kinins: Cardiovascular, Biochemical, and Neural Actions. Plenum Press. pp. 57-64 (1970).

Beaven, V.H., Pierce, J.V., and Pisano, J.J.: A sensitive isotopic procedure for the assay of esterase activity: measurement of human urinary kallikrein. Clin. Chim. Acta 32: 67-73, 1971.

Serial No. NHLI-110(c)
1. Experimental Therapeutics Branch
2. Section on Neuroendocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Steroid Hormones, Protein Hormones and Electrolytes on Neural Function.

Previous Serial Number: NHLI-166(c)

Principal Investigator: Robert I. Henkin, M.D., Ph.D.

Other Investigators: M. Buchsbaum, M.D.
J. Fontana, Ph.D.
C. Gillen, M.D.
N. Jacobs, M.D.
M. D. Walker, M.D.

Cooperating Units: Department of Medicine, University of Cincinnati School of Medicine, Cincinnati, Ohio; National Cancer Institute, Baltimore, Maryland; Polytechnic Institute of Brooklyn, Brooklyn, N. Y.; National Institute of Mental Health, Bethesda, Md.

Project Description:

Objectives: To investigate systematically the interrelationships between steroid hormones, protein hormones and electrolytes on neural function with respect to sensory detection and recognition and to investigate the mechanisms by which these interrelationships occur.

Major Findings: 1. Effects of adrenocorticosteroids on NE and Ch uptake of brain synaptosomes. It has long been known that the excess of and lack of carbohydrate-active steroids (CAS) greatly affect central nervous system activity. The mechanism by which CAS influences CNS activity remains relatively obscure. It appears however that one possible mechanism may involve an interaction between the steroid molecules and the actual nerve endings. The object of this investigation is to study the possibility of this

interaction.

In light of the difficulty involved in examining a steroid-nerve ending interaction in vivo, synaptosomes (pinched off nerve endings consisting of intact nerve membranes enclosing synaptic vesicles) were utilized since these particles provide a convenient in vitro system for the study of the effects of drugs on neurotransmitter function. Therefore, the effect of CAS on the synaptosomal uptake and release of various neurotransmitters was investigated. Cortisol hemisuccinate was chosen as the adrenal steroid although it does not possess high CAS activity. However, it is water soluble and allows a completely aquaeous system to be employed.

Active uptake of NE and Ch by synaptosomes was demonstrated. Cortisol hemisuccinate inhibited NE and Ch uptake, and results are summarized below:

Inhibition by Cortisol Hemisuccinate of NE Uptake

Concentration of Steroid	% Inhibition of NE Uptake
0.8×10^{-4} M	0
2.0×10^{-4} M	2.6
4.0×10^{-4} M	3.4
8.0×10^{-4} M	4.8
1.6×10^{-3} M	13.2
3.2×10^{-3} M	18.2
6.4×10^{-3} M	43.3
1.2×10^{-2} M	62.0

Inhibition by Cortisol Hemisuccinate of Ch Uptake

Concentration of Steroid	% Inhibition of Ch Uptake
4×10^{-4}	0
8×10^{-4}	2.8
1.6×10^{-3}	3.8
3.2×10^{-3}	26
6.4×10^{-3}	61

Inhibition by cortisol hemisuccinate of NE and Ch uptake required high concentrations of steroid.

It has been postulated that part of mechanism concerned with neurotransmitter uptake involves ATPase activity. It was determined that our synaptosomal preparations contained appreciable Na-K ATPase activity (See Table).

Fraction	Total Activity of Na-K ATPase
Nuclei + cell debris	22.5 Units
17,000g Supernate	2.1 Units
Synaptosome	7.0 Units

Therefore, the effect of cortisol 21 hemisuccinate on synaptosomal Na-K ATPase activity was investigated. The results are summarized below:

Concentration of Steroid	% Inhibition of Na-K ATPase Activity
5×10^{-4} M	18
1×10^{-3} M	23
2×10^{-3} M	35
4×10^{-3} M	44
8×10^{-3} M	58

Thus, cortisol hemisuccinate inhibited Na-K ATPase activity and this inhibition took place at steroid levels which also inhibited NE and Ch uptake.

2. EEG changes in man in the absence of adrenocorticosteroid secretion and during administration of ACTH. These studies comprise two major efforts; one, the effect of these changes on visual evoked response activity (AER) in man, the second, the effect of these changes on sleep phenomena in man. In the first part of this study patients with several forms of adrenal cortical insufficiency had measurements made of their visual evoked responses to graded light intensities before and during withdrawal from the effects of their replacement therapy. In addition, these measurements were made during administration of ACTH. Results of these studies indicate that during ACTH administration there is significant enhancement of the AER which is dependent of the ACTH effect on the adrenal gland and appears to be a direct effect of ACTH on brain activity. In the second part of this study, the sleep activity of patients with various forms of adrenal cortical insufficiency was measured under the same conditions as noted in part one. Results of these studies indicate that REM sleep decreases significantly during the time which replacement therapy is withdrawn, is further decreased during ACTH administration and return to normal during readministration of steroid hormone therapy. These studies differ from those carried out in normal volunteers which indicate that administration of carbohydrate-active steroids is correlated with increases rather than decreases in REM activity.

3. Distribution and metabolism of H^3 steroids in nervous tissues of the cat. In continuation of previous studies the distribution of H^3 testosterone and progesterone in various tissues of the eviscreted cat has been studied. Following a controlled rate infusion of H^3 testosterone concentrations of hormone in the brain followed a pattern similar to that previously shown for H^3 cortisol but quite different from that of H^3 progesterone. The metabolites of H^3 testosterone in the tissues studied are being measured by gas-liquid chromatography by Dr. Leon Sholiton, University of Cincinnati.

4. EEG changes in patients with serum calcium abnormalities and with pseudohypoparathyroidism. The effects of serum calcium concentration on the visual average evoked responses (AER) was studied in 24 patients with various disorders of calcium metabolism

and in 6 normal volunteers given 450 U of parathyroid extract for 4 days. Low serum calcium concentrations (below 8 mg/100 ml) were associated with greater amplitude and shorter latency AERs than were high serum calcium concentrations (above 12 mg/100 ml). Administration of parathyroid extract in doses that raised serum calcium concentration, albeit minimally, had no effect on the AER latency or amplitude. AER latency and amplitude changes were correlated with serum calcium concentration rather than with the patients' diagnostic category.

Perceptual function in 8 patients with pseudohypoparathyrodism (PHP) was studied using two neurophysiological measurement techniques, the average evoked response (AER) and motor nerve conduction velocity. A battery of psychophysical tasks including reaction time, size estimation, hidden pictures and the rod and frame procedure were also used. Patients with PHP had significantly longer latency visual AER and slower reaction times than did a group of normal volunteers and the patients performed erratically and poorly on the psychophysical tasks. Differentiating these patients from patients with diffuse mental deficiency were two relatively specific perceptual response patterns: (1) AER amplitude decreased with increasing stimulus intensity and (2) reaction time showed abnormally strong effects of the duration of the preparatory interval. These results could not be attributed to alterations in serum calcium concentration. Each of these patients did exhibit decreases in cyclic AMP in the urine. Since cyclic AMP appears to play some role at synaptic junctions it may be useful to speculate about the role which cyclic AMP may play in sensory function in the future. Future studies are designed to investigate this possible relationship.

5. The role of adrenocorticosteroids in neural function. During this past year work in laboratory has focused on one sensory modality, taste, and the effects of removal and replacement of carbohydrate-active steroids on taste responses in man. Two general kinds of measurements were made, threshold measurements and intensity measurements. We tried to relate these two types of measurements to each other and to show the effects of adrenal corticosteroids on each. Based upon observations in man we derived a taste equation which appears to fit the taste responses of normal man and of patients with various forms of adrenal cortical insufficiency and Cushing's syndrome in their treated as well as untreated state.

This equation is:

$$I/I_{max} = \frac{K\ (T)^2}{K\ (T)^2 + 1}$$

where I is the intensity of tastant at concentration (T), I_{max}, the maximum intensity at highest (T) and K is a constant. Based upon our previous data and hypotheses there is a specific interaction between tastant and receptor molecule at the taste bud membrane such that a tastant-receptor complex is formed (RT). If we assume that one tastant reacts with one receptor molecule in a simple equilibrium it is possible to derive the taste equation 1 shown above as follows:

$$R + T \underset{\underline{K_1}}{\overset{K_1}{\rightleftharpoons}} RT \qquad K = K_1/\underline{K_1} \qquad \text{eqn 2}$$

and

$$\frac{(RT)}{(R) + (RT)} = \frac{K\ (T)}{K\ (T) + 1} \qquad \text{eqn 3}$$

The forced scaling intensity data in the normal volunteers, and to a lesser extent in the patients, was described by eqn 1 which is

$$I/I_{max} = \frac{K\ (T)^2}{K\ (T)^2 + 1}$$

The binding stoichiometry $R + 2T \underset{\underline{K_1}}{\overset{K_1}{\rightleftharpoons}} RT_2$ $K = K_1/K_1$ eqn 4

$$\frac{(RT)_2}{(R) + (RT)_2} = \frac{K\ (T)^2}{K\ (T^2)^2 + 1} \qquad \text{eqn 5}$$

would lead to eqn 1 if one assumes that the intensity is proportional to the concentration of RT complexes. In this model the single parameter K obtained by fitting eqn 1 to the experimental data is the binding constant for the formation of the RT_2 complex. Equation 1 can be derived as follows: At equilibrium, eqn 4 becomes $(RT_2) = K\ (R)\ (T)^2$. Now $(R) + (RT_2) = R_0$ where R_0 is the total number of receptor molecules present. Therefore,

$$(RT_2) = K\left[R_0 - (RT)_2\right](T)^2$$

$$(RT_2) = \frac{KR_0\ (T)^2}{K\ (T)^2 + 1}$$

and if we assume that the intensity is proportional to the number of (RT_2) complexes, i.e., $I = C(RT_2)$, then

$$I = \frac{CR_0 K (T)^2}{K (T)^2 + 1} \qquad \text{where}$$

C is a proportionality constant. As (T) becomes very large, I approaches its maximum value $I_{max} = CR_0$. Therefore,

$$I/I_{max} = \frac{K (T)^2}{K (T)^2 + 1}$$

which is eqn 1. Eqn 4 is of course only one of many possible models from which eqn 1 can be derived. However, from the simplest model (eqn 2) eqn 1 cannot be derived.

This model would explain why the intensity function reaches a maximum at high tastant concentrations; the tastant molecules would complex with all the available receptor molecules and introduction of more tastant could not form more complexes. As the tastant concentration decreases the number of receptor molecules complexed would also decrease and at a sufficiently low concentration the signal produced would no longer be recognized, albeit detected, and if tastant concentration is decreased further, it would not be detected from background.

The signal produced by this complex formation presumably occurs at the taste receptor and is anatomically, spatially and temporally separated from the neural events (Fig. 6). Thus, the classification of pre-neural and neural events has physical bases.

CAS deficiency and excess states may affect the preneural events of taste by affecting the binding constants by which tastant-receptor molecule complexes occur or by affecting the stoichiometry of binding. Excessive and inadequate amounts of CAS decrease the binding constants for all taste qualities because the intensity curves are all shifted to higher concentrations. From the second point of view the possible deviation of the data from eqn 1 toward a lower order (in (T)) equation such as eqn 3 would imply that the stoichiometry of binding is altered. Apparently there is a concentration of CAS at which the intensity is maximal for a given tastant concentration and either an excess or a deficiency of CAS lowers the intensity.

The fit of equation 1 to the data suggests that two tastants are required for the recognition process to occur normally. The equation does not specify the spatial relationships among the tastants and receptors. However, these relationships are suggestive of the existence of cooperative binding in which K would be the product of the square (Ki^2) of the intrinsic binding constants (Ki) of the tastants and a coupling term Kc; i.e., $K = Ki^2Kc$. According to this interpretation excess or deficiency CAS states would shift the intensity curves by reducing the coupling constant which would, in the limit of Kc = 1, also shift the binding stoichiometry to a lower order. High tastant concentrations can overcome this decoupling by sheer mass action.

Significance: 1. Role of adrenocorticosteroids in neural conduction. We have shown that NE and Ch uptake by brain synaptosomes are markedly inhibited by the presence of carbohydrate-active steroids. This effect appears to be mediated by inhibition of Na-K activated ATPase activity by carbohydrate-active steroids. Since no effect of pharmacological doses of these steroids could be observed on the Na or K currents of the nerve it is clear that the effect of these corticosteroids on neural function is not on the excitable portion of the nerve membrance, per se, but rather on the metabolism of the nerve; i.e., in maintaining the membrane potential and also on synaptic conduction. Whatever effect these steroids may have on axonal conduction is probably through their effects on myelin.

2. Role of adrenocorticosteroids in neural function. We have described an equation by which taste phenomena occur in normal man and in patients with various abnormalities of adrenal cortical function. This equation is:

$$I/I_{max} = \frac{K\ (T)^2}{K\ (T)^2 + 1}$$

where I is the intensity of tastant at concentration (T), I_{max}, the maximum intensity at highest (T) and K is a constant. This equation can be derived from simple relationships of the theory which we have developed over the past 3 years. The model which is described by this equation would explain why intensity functions in taste reach a maximum at high tastant concentrations; taste molecules would complex with all available receptor molecules and introduction of more tastant could not form more complexes. De-

ficiency and excesses of carbohydrate-active steroids in man may affect the pre-neural events of taste by directly affecting the binding constants by which tastant-receptor molecule complexes occur or by affecting the stoichiometry of binding. Our data demonstrate that excessive and inadequate amounts of these steroids decrease the binding constants for all taste qualities because the intensity curves are all shifted to higher concentrations. Apparently there is a concentration of steroid at which intensity is maximal for a given tastant concentration and either an excess or a deficiency of steroid lowers the intensity.

3. Role of calcium in EEG. Through the application of the quantitative technique of visual averaged evoked responses to clinical investigative problems it has been possible to study the EEG changes in man during several physiological states involving calcium metabolism. Our studies have shown that low serum calcium concentrations (below 8 mg/100 ml) are associated with greater amplitude and shorter latency AER than high serum calcium concentrations (above 12 mg/100 ml).

Proposed Course of Project: Specific work will be carried out which will identify the role which carbohydrate-active steroids play at the synapse through their effects on several neurotransmitter agents. In man, the theories and hypotheses made during this past year with respect to the effects of these steroids on taste will be tested and evaluated.

Honors and Awards: None

Publications:

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Serial No. NHLI-111(c)
1. Experimental Therapeutics Branch
2. Section on Neuroendocrinology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Trace Metal Metabolism

Previous Serial Number: NHLI-164 (c)

Principal Investigator: Robert I. Henkin, M.D., Ph.D.

Other Investigators: Joseph Fontana, Ph.D.
Eugene Giroux, Ph.D.
N. Prakash, Ph.D.
R. Michell, Ph.D., D.V.M.
J. Pierce, D.V.M.

Cooperating Units: Department of Chemistry, Charlottesville, Va.
Department of Medicine, Presbyterian Hospital, N.Y., N.Y.

Objectives: This project was designed to study the physiology, metabolism, biochemistry and pathology of copper, zinc and other trace metals in physiological fluids and tissues of normal subjects in patients with various diseases and in animals. We have also studied the interaction between metals and their binding proteins.

Major Findings: Physiology

1. Copper depletion and corticosteroid production in the adrenal gland of cats and rats. The Δ^5-3 ketosteroid isomerase activity in guinea pig adrenals is dependent on the presence of copper ions. This enzyme activity along with 3 β-hydroxysteroid dehydrogenase activity is responsible for the conversion of pregnenolone to progesterone in the early biosynthesis of nearly all biologically active steroid hormones. In conjunction with our laboratory's interest in the interrelationship of trace metals and normal body function, the effect of copper deficiency in cats and rats on adrenal steroid production was investigated.

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Adrenals obtained from normal and copper deficient cats and rats were sliced and incubated with tritiated cholesterol. In one experiment involving rat adrenals, pregnenolone, progesterone, corticosterone and cortisol were isolated, using paper and thin layer chromatography. In a second experiment involving cat adrenals, cortisol, cortisone and corticosterone were isolated, again using paper and thin layer chromatography. Final identification of all compounds was made by successive crystalizations to a constant specific activity using authentic carrier.

The effect of copper deficiency on adrenal weight is described in Table I.

TABLE I

Experiment	Wet Weight of Adrenals	
	Copper Deficient	Normal
Cats (3)	177.4 ± 9.5 (s.e.m.)*	102.8 ± 4.3 (s.e.m.)*
Rats (6)	93.0 ± 4.3 (s.e.m.)**	64.0 ± 7.7 (s.e.m.)**

* Each adrenal compared.
** Each pair of adrenals from a rat compared.

These results indicate that copper deficiency results in a marked adrenal hyperplasia.

The results of the incubation experiments indicate that copper depleted animals produce markedly reduced levels of cortisol, cortisone and corticosterone when incubated with H^3 cholesterol and compared to adrenals of normal animals (Table II), but both groups produce similar levels of pregnenolone (Table III).

TABLE II

Type	Corticosterone*	Cortisol*	Cortisone*
Normal-1	30,926	6,318	6,690
Copper Depleted-1	10,083	3,813	3,778
Normal-2**	91,709	14,370	--
Copper Depleted-2**	32,166	5,362	--
Normal-3	12,010	3,267	--
Copper Depleted-3	7,568	2,452	--
Normal-4**	19,098	13,149	--
Copper Depleted-4**	7,601	2,744	--
Normal-5	13,246	14,593	--
Copper Depleted-5	8,888	2,584	--
Normal-6**	27,252	12,972	--
Copper Depleted-6	6,900	3,319	--

*dpm/gm tissue, net weight
**$Cu(NO_3=)$ 1 x 10^{-5} M added to reaction vessel

TABLE III

In Vitro Steroid Production by Adrenals from Normal and Copper Deficient Rats

	Cortisol*	Corticosterone*	Pregnenolone*	Progesterone*
Copper Deficient(5)	31,234±7,295	42,133±4,476	75,112±26,600	50,197
Normals(5)	48,609±6,277	76,614±21,138	84,182±29,437	185,014

*gpm/gm tissue, net weight

These results indicate that the block in steroidogeneses caused by copper depletion occurs at the conversion of pregnenolone to progesterone, at the Δ^5-3 hydroxysteroid-dehydrogenase-isomerase step.

2. Metal-protein complexes in physiological fluids. This project was designed to quantitate the various metal-ligand complexes which occur in physiological fluids. Earlier work involved fractionating serum into stable metalloproteins and relatively easily dissociable metal complexes could not be determined. We were interested in the manner by which various trace metals formed complexes with protein or peptide moeities and we have studied these complexes in terms of their ability to pass a variety of semi-permeable membranes.

Urine was examined initially. It was stored at 4°C, then warmed to 37°C, brought to pH 5.0 with HAc, centrifuged and filtered. The filtrate was passed through membranes of nominal pore sizes 46, 38, 30 and 24 A° diameter. The apparatus and membranes for carrying out these procedures came from the Amicon Corp., Boston, Mass. All metal measurements were made by atomic absorption spectrophtometry or flame spectrophotometry. Only the smallest membrane significantly retained calcium, magnesium and zinc complexes. These results indicated that metals are bound to small ligands in urine. In an effort to evaluate the character and size of these ligands model studies of the interraction of metal complexes with known ligands are in progress, as noted below.

In combination with gel filtration studies it is possible to identify amino acid, carboxylate and inorganic acid anion ligands; however, because of their lack of homogeneity it may not be possible to quantitatively describe the metal distribution among these ligands. These studies can be carried out easily in blood and we are in the process of doing so. We are also evaluating the manner by which metals pass these membranes in conjunction with the assays of various protein, polypeptides and peptides in the ultrafiltrate and retentate which should lead to kinetic information related to metal distribution. This information has particular relevance to the manner by which protein-metal complexes cross the glomerulus of the kidney.

3. Metal-protein complexes in tissue. Kidney and liver contain sulfhydryl rich polypeptides which are capable of binding zinc, copper, mercury, cadmium and other metals. The character of the polypeptide and the metals which it contains appear to

differ in different tissues and in different animal species. Vallee has stated that metallothionein is a cadmium containing polypeptide and he has suggested that this protein plays a role in metal detoxification in the kidney. Horse and human kidney are rich in cadmium and are excellent sources of metallothionein.

Our results indicate that dog kidney contains about 15 ppm Zn, 3 ppm Cu and less than 0.2 ppm cadmium per gm wet weight of tissue. Valle's data from human kidney cortex indicates there is 350 ppm Zn and 140 ppm Cd per gm dry weight of tissue. Purified human kidney metallothionein contains 3.7-3.9 g-atoms Cd, 3.2-4.3 g-atoms Zn, 0 g-atoms Cu and 0.3 g-atoms Hg. In a preliminary experiment dog kidney cortex was homogenized in dilute phosphate buffer and the homogenate prepared in a manner similar to that used by Vallee for preparation of metallothionein. Various fractions were filtered through a Sephadex G-75 column. Two low molecular weight metalloproteins were observed. One, MW 34,000, with equal amounts of copper and zinc, is probably the cytocuprein described previously by Carrico and Deutsch. The second, MW 10,000 corresponds to metallothionein in terms of size, but contains Cu without any Zn or Cd. This metalloprotein will be isolated and characterized in future studies, its sulfhydryl content determined and its avidity for various metal ions identified.

In a similar manner protein complexes with zinc and copper in tongue of rat are being identified and characterized.

4. Circadian variation of copper and zinc in man. Copper and zinc metabolism was studied in ten normal volunteers on a constant regimen consisting of an alternate 4 hourly intake of a liquid diet or distilled water and a regulated amount of physical activity. Urine was collected in 4 hour periods and blood samples drawn in the middle of these periods. A circadian pattern of variation for serum copper and zinc concentration was demonstrated; serum copper was above the mean at 10:00 am and 2:00 pm, at the mean at 6:00 pm and 10:00 pm, and below the mean at 2:00 am and 6:00 am. This pattern persisted in 2 subjects who received 2.5 mg prednisolone every 6 hours for 3 days. A circadian pattern for the urinary excretion of copper but not for zinc was demonstrated. Serum ceruloplasmin tended to follow serum copper concentrations and suggested that the regulation of metal binding proteins may be important in the circadian pattern of these metals observed in serum. To our knowledge this is the first demonstration of a circadian pattern of variation in ceruloplasmin.

Data were collected over a period of 5 days in each subject studied. We are now in the process of analyzing these data over this time period to determine whether or not a longer cycling process occurs and whether phase relationships between changes in blood and urine can be determined.

5. Pituitary-gonadal regulation of copper and zinc metabolism in the female rat. This study was undertaken to specify the changes in copper and zinc concentrations in serum of female rats during various physiological states: (1) during the estrus cycle, (2) during pregnancy, (3) during pseudopregnancy, (4) following castration, (5) following castration and hypophysectomy and during administration of the pituitary gonadotropins LH and FSH, and (6) following castration and during administration of estrogen, progesterone or estrogen and progesterone together. Results of these studies indicate that there is cyclic pattern of change of both copper and zinc during the estrus cycle with serum copper and zinc concentrations both reaching a peak at the time of estrus coincident with ovulation. During pregnancy serum copper concentrations increase until day 15, then decrease thereafter, again following the pattern of estrogen secretion. Serum zinc does not change during this early period, but falls dramatically during the last days of pregnancy. Post-partum serum copper concentrations fall precepitously to values below those of castrated animals, but return to estrus levels within 5 days of parturition. Zinc concentrations in serum increase immediately post-partum to estrus levels and remain there throughout the post-partum period. Following the production of pseudopregnancy by means of vaginal manipulation of the rat at an appropriate time during estrus there is a significant increase in serum concentration of copper and of zinc, with the zinc increase lagging the copper increase by 6 days. This condition demonstrates that changes in serum copper and zinc concentrations in the rat are significantly influenced by endogenous progesterone secretion as well as by estrogen secretion. Following castration there is a significant decrease in serum copper concentration and an increase in serum zinc concentration. This could be due to the lack of estrogen and progesterone or to the effects of LH and FSH. To evaluate this concent overiectomized, hypophysectomized rats were treated with LH and FSH. There was no effect of either hormone alone on serum copper or zinc concentrations in these animals. Administration of estradiol 17 beta to castrated female rats resulted in a dose-response increase in serum copper without any change in serum zinc. Administration of progesterone resulted in similar increases in serum copper and decreases in serum zinc although the dose response relationships

noted with estradiol administration did not occur. Administration of both progesterone and estradiol 17 beta produced an enhancement of both effects with a significant increase in serum copper concentration and a significant decrease in serum zinc concentration.

These results demonstrate that copper and zinc metabolism in the rat are in part controlled by estrogen and progesterone and that the concentrations of these metals in the serum change in association with changes in either or both of these hormones. The role of these metals in these physiological processes are at present being investigated as follows: (1) the role of copper is suggested as one of the controlling factors initiating ovulation in the rat, (2) estrogen and progesterone can produce changes in copper and zinc metabolism in male rats.

6. Copper and zinc metabolism in female sheep and cows. In a study similar to that noted above in the rat, but much less extensive changes in serum concentrations of copper and zinc in sheep and cow were evaluated during pregnancy, post-partum and following administration of estrogen. Distinctly different from results in rat or man no changes in either copper or zinc metabolism were observed in sheep or cow during pregnancy, post-partum or following administration of estrogen. These studies will be extended by administration of progesterone to these same animals.

7. Effects of metals on norepinephrine (NE) and choline (Ch) uptake of brain synaptosomes and on adenosinetriphosphatase activity. Because of the interrelationships between steroid synthesis and metal cofactors noted above and because of interrelationships between this system and neural conduction which we have suggested we investigated the interrelationships between metals and uptake of NE and Ch on brain synaptosomes and ATPase activity. Using established techniques the following effects on activity have been demonstrated using various physiological concentrations of metals:

METAL	Uptake (%)* Ch	NE	Activity (%)* ATPase
Zn	20	40	10
Ne	70	80	40
Co	80	20	50
Cu	0	0	0
Hg	0	0	0
Mn	60	120	30
Sn	110	300	150

* Results indicate % uptake and indicate activity remaining at highest concentration of metal ion used.

These results demonstrate that most metals inhibit both Ch and NE uptake and ATPase activity but to varying degrees. The manner by which this inhibition occurs is being investigated. Two metals Cu and Hg are extremely toxic to Ch and NE uptake and ATPase activity whereas two other metals Mn and Sn appear to enhance the uptake of NE while Sn appears to enhance ATPase activity. The results of Sn in this system are specific for Na-K dependent ATPase activity having no effect on Mg-activated ATPase activity. These results are being confirmed in further experiments.

8. Effects of hormones of the adrenal cortex on metal concentration in various tissues of the cat. We have previously demonstrated the interrelationships between steroid hormone synthesis and copper. In these studies we have looked at the converse problem; i.e., in the presence of too little or too much secretion of the adrenal cortex, what are the effects on metal concentration in several tissues of the cat? Cats were adrenalectomized and maintained for 3-5 weeks at which time they were exsanguinated and their tissues removed using special techniques. Control cats were sacrificed in parallel with the adrenalectomized cats. Brain, spinal cord, sciatic nerve, liver and sciatic muscle mass of both groups of cats were analyzed for the concentration of approximately 25 metals by spark-source mass spectrometry. Results of these studies indicate that concentrations of most trace metals increase

in the liver following adrenalectomy whereas concentrations of these metals in brain are either unchanged or decreased. Changes in spinal cord, sciatic nerve and muscle mass generally follow the pattern observed in liver. Administration of excessive doses of both carbohydrate-active and Na-K active adrenal corticosteroids to adrenalectomized cats demonstrated that metal concentration may be decreased significantly in liver, spinal cord, sciatic nerve and muscle mass. These studies indicate that concentrations of metals in several tissues are dependent upon the endogenous secretion of hormones of the adrenal cortex.

9. Uptake of Zn^{65} by various tissues of the rat. Ten μc of Zn^{65} were injected into rats and its distribution evaluated in terms of % dose found in physiological fluids and tissues from minutes to 7 days after injection. In general, blood levels rapidly fell within the first 24 hours after injection and uptake was highest in bone, liver and tongue, respectively. Separating the tongue into an anterior portion, containing fungiform papillae and taste buds, and posterior portion, containing foliate and vallate papillae and taste buds, indicated that the posterior portion contained more Zn^{65} than the anterior portion. Many more taste buds are present in the posterior portion than in the anterior portion.

Significance: During the past year we have systematically investigated several aspects of the physiology and biochemistry of metal metabolism in mammalian systems.

1. Metals and steroidogenesis: We have extended our work of last year and designated that the Δ^5-3 hydroxysteroid-dehydrogenase-isomerase step in steroidogenesis in rat and cat requires copper. We have also shown that the concentrations of metals in several tissues are dependent upon the presence of the endogenous secretion of the adrenal cortex and that these effects may differ among various tissues. The liver appears to be an important source of all metals and that these metals are mobilized by increasing the amount of circulating adrenocorticosteroids. Metal concentration in brain is not controlled in the same manner. These studies have great importance to the manner by which tissues take up, store, and metabolize metals.

2. Metal-protein interactions: We have demonstrated that metals in urine are not "free" in the sense of appearing in urine as ions but are liganded with relatively small molecular weight peptides or proteins. Two distinct metal containing proteins

have been identified in dog kidney. One is of MW 34,000, contains equal amounts of copper and zinc and is probably cytocuprein. The other is of MW 10,000, corresponds to metallothionein in size but contains only copper, without zinc or cadmium. These latter studies indicate that kidney metallothionein may differ significantly in different animal species, may contain different metals and may not contain cadmium.

3. Metal-neurotransmitter interactions: We have initiated studies in this area and we have demonstrated that metals, in general, are inhibitory for the uptake of NE and Ch by brain synaptosomes and for the activity of both Na-K activated and Mg activated ATPase activity. The specific nature of this inhibition has been systematically investigated and related to the nature of the metal-neurotransmitter complex. However, two metals Mn and Sn appear to enhance norepinephrine uptake. The mechanisms by which this enhancement occurs has been systematically investigated. Sn acts to enhance Na-K ATPase activity specifically without effect on Mg-ATPase activity. It is through this effect that it enhances the uptake of NE by brain synaptosomes. These studies suggest the first physiological role for Sn in mammalian systems.

4. Pituitary-gonadal regulation of copper and zinc metabolism in the female rat. These studies have demonstrated that serum concentrations of copper and zinc are influenced primarily by both estrogen and progesterone, not by LH or FSH. The relationship between copper and the initiation of ovulation has been investigated. The role of copper in the control of fertility in man is of particular importance since intrauterine devices coated with copper are more effective for several reasons than the same devices coated with Teflon. The mechanism for this phenomenon is not yet apparent.

5. Circadian variation of copper and zinc in man. We have established that serum concentrations of copper and zinc exhibit circadian changes in man and that these changes can be observed for copper in urine but not for zinc. A circadian variation for ceruloplasmin in serum has also been observed for the first time and this may relate to the circadian changes observed for the first time and this may relate to the circadian changes observed in serum copper concentrations. These changes are not abolished following blocking of the endogenous secretion of the adrenal cortex and of pituitary ACTH for a period of 3 days.

6. Role of metals in taste. Injection of Zn^{65} in rats demonstrated that the tongue was one of the most active tissues in accumulating Zn after bone and liver. Correlating these studies with those previously noted in which zinc was found in the epithelial layer of papillae by laser microprobe spectroscopsy suggest that taste bud bearing papillae and perhaps even taste buds themselves may take up zinc avidly.

Proposed Course of Project: Each of the studies outlined will be continued in the specific manner indicated. In general, we will investigate the physiology and biochemistry of metal-protein complexes in several physiological fluids, the nature of the role of Sn and Mn in enhancement of NE uptake by synaptosomes and the role of Sn in ATPase activity. We will continue to investigate the role which metals play in neural activity. These studies will fit closely with the role which metals play in the taste process. In addition, the role of taste buds and other tissues in the uptake of Zn^{65} and the role of various factors which influence the uptake, metabolism and storage of this metal will be evaluated in man and other animals.

Honors and Awards: None

Publications:

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1. Experimental Therapeutics Branch
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3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Taste and Olfaction

Previous Serial No.: NHLI - 167 (c)

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Project Description:

Objectives: To investigate, in a systematic manner, the anatomical, physiological, pharmacological and pathological correlates of taste and olfaction.

Major Findings: Taste

Anatomy. In order to understand the physiology and biochemistry of taste, the taste receptors must be identified and their function specified. The correlation of form and function in taste buds of any vertebrate or invertebrate system at present is rudimentary. In order to identify and specify the taste receptors we have undertaken the following projects:

1. Do taste buds in different papillae in man and animals differ in form or function? In man, in order to answer this basic question taste buds from fungiform, circumvallate and palatal papillae in normal man were studied systematically by light and electron microscopy. Because of the size and location of the buds in papillae their orientation is difficult. Knowledge of taste acuity in those subjects or animals in whom taste buds are studied anatomically is essential for evaluation of normality. These two aspects of anatomical studies of taste buds have been carried out.

Systematic evaluation of taste buds in man has been made in patients who have had their taste acuity tested and found to be normal. These patients are usually to undergo a surgical procedure in the National Cancer Institute. With their informed consent, at the time of surgery, fungiform, circumvallate and palatal papillae are excised, immediately placed in glutaraldehyde and embedded in plastic and fixed for sectioning. Thick sections are cut (less than 1μ) and first evaluated by light microscopy to insure the presence of buds and their proper orientation. This has saved weeks of time previously spent hunting for buds through the use of traditional thin sectioning techniques. After the bud is properly oriented thin sections are made and the bud evaluated in detail. In this manner buds from approximately five circumvallate papillae have been studied in detail and they confirm our earlier observations of the anatomical organization of the bud; i.e., from the pore inward there are 8 levels of organization; (1) a pore filled with granules and finger-like processes of Type I cells, (2) the cell process layer, (3) the dense extracellular layer, (4) the layer of neurosecretory granules, (5) the kinetosomal layer, (6) the nuclear layer, (7) the synaptic vesicle layer and (8) the axonal layer. There are 4 anatomically distinct types of cells identifiable in this taste bud as follows: Type I cells-- these comprise at least 80% of the cells of the bud, have processes which extend out of the bud into the open pore have paired kinetosomes, have neurosecretory granules, have their synapses such that they appear to be afferent to the central nervous system and appear to be the receptor cells of the taste bud; Type II cells--these comprise about 10-15% of the cells of the bud, have processes which extend near the pore but not necessarily into the pore, have centrioles adjacent to the cell nuclei but not kinetosomes, have vacuolated cytoplasm, do not have neurosecretory granules, have their synapses such that they appear to efferent from the CNS, have large fiber bundles which run from

below the nuclear area to the infra-pore area and appear to be the effector cell which participate in the closure of the bud; Type III cells--these comprise 1-5% of the cells of the bud, have processes which extend into the pore but other characteristics including their possible function are not well established; Type IV--these cells have been seen only occasionally in the bud, are located near the base of the bud and their anatomy and function are not well evaluated. In a more preliminary manner, two buds from fungiform papillae have been evaluated. Only two cell types, Type I and Type II cells, have been observed in them. They differ in anatomy from the buds of the circumvallate papillae due to their long necks and their opening directly into the oral cavity. The buds are located on the lingual surface of the papillae as opposed to the position of the buds of the circumvallate papillae which are in crypts beneath the lingual surface. Palatal papillae possess buds which are more like buds from fungiform than circumvallate papillae since they open into the oral cavity being positioned on the palatal surface of the papilla.

In the rat, this same anatomical framework has been observed in the vallate papillae containing buds similar to the circumvallate papillae in man. Similar placement of buds are in fungiform and palatal papillae containing equivalent structures.

It is of interest that no blood vessels or lymphatic channels were observed in any taste bud. Similarly, no mitotic figures were clearly identified which raises the questions of how the taste bud maintains itself, from which tissues do taste cells form, in what manner do taste cells degenerate and how does the bud handle degenerating material. These questions have not yet been approached systematically although estimates suggest that the cells of the rat taste bud regenerate over a period of 3-11 days.

2. What specialized sensory receptors, if any, exist in the papillae themselves and, if they exist, can they be related to the taste process in any manner? In order to answer this question a systematic evaluation of the anatomy of the fungiform, circumvallate and palatal papillae in man and also the valate papilla in rat has been undertaken independent of the study of the taste buds. These studies produced results not previously obvious due to the technique by which they were carried out. Since all tissues were fixed in glutaraldehyde, little artifactual damage to the tissue was made in comparison to usual techniques of fixing in

formalin and embedding in parafin as earlier anatomists had done. Sections from the tissues used in the present study were stained with osmium. In these tissues myelinated fibers could be demonstrated extending into the epithelial layer of the papillae adjacent to the taste bud in circumvallate papillae of man and valate papillae of rat. These fibers, not previously described, may subserve touch, temperature and pain in the papilla. In addition, Paccinian corpuscles (specialized pressure receptors) were demonstrated for the first time. Meissner's corpuscles and other specialized sensory receptors and nerve endings were also observed. These structures indicate that the papillae of the tongue contain the same sensory receptors as does the epithelial layer of the skin. In addition, striated muscle has been observed to be present at the base of each papilla and fibers from major muscle bundles of the lingual muscles appear to branch off to each papilla. No specific connections between the striated muscle fibers and the Type II cells have yet been clearly observed.

3. What differences, if any, exist between the taste buds of patients or animals with altered taste acuity and those with normal taste acuity? The taste receptor is the only receptor (other than touch) that can be anatomically evaluated serially in patients with a sensory abnormality without limitation to subsequent sensory function. For this reason taste buds from circumvallate papillae in patients with idiopathic hypogeusia, aglycogeusia and known vitamin A deficiencies have been evaluated and compared to taste buds from individuals with normal taste acuity. The changes observed in buds from patients with idiopathic hypogeusia previously described were confirmed to be localized mainly in the pore region of the bud with disruption of the normal organization, as noted in this report of 1970.

Preliminary results from the bud of the one patient with aglycogeusia studied demonstrated marked degeneration of the Type II cells with a perinuclear hyalinized appearance. No processes of Type I cells could be observed in the pore region but rather club-shaped clumps of cytoplasm.

Taste buds from circumvallate papillae from prisoners before, during and after feeding a diet deficient in vitamin A were excised and studied in detail. Taste and smell acuity was also measured before, during and after feeding the diet at the time of the biopsies. Tissue was handled as noted previously. When these men were vitamin A deficient they exhibited several ab-

normalities of vitamin A metabolism: skin abnormalities, abnormal dark adaptation curves and abnormal electroretinograms. Buds from men who were vitamin A deficient showed disorganization of the pore area with absence or marked decreases in the finger-like processes of the Type I and II cells, marked decreases of the dense extranuclear material and the presence of large "lipid inclusion" bodies which have not been previously observed in any bud. Although these patients exhibited marked hyperparakeratosis of their skin, the papillae samples were not particularly keratinized nor were the pores of the taste buds covered by keratin. In fact, the pore areas were clearly open to the crypt of the papilla. The number of taste buds present in the papillae, however, were estimated to be decreased below normal by more than a factor of 2. Kinetosomes were present in Type I cells as were neurosecretory granules; the latter were of normal number and stratification. Type II cells were normal in number and appearance without any hyalinized perinuclear area. The abnormalities noted appear to be different from any we have yet seen. In following the anatomy of the buds after vitamin A replacement using each patient as his own control, the abnormalities noted during the depleted state disappear and the buds cannot be distinguished from those of other normal subjects. Taste acuity was also measured in these patients after repletion with vitamin A and found to be normal as were other function of vitamin A metabolism including skin appearance, grossly and by histological examination and visual function.

4. Is it possible to find an animal whose taste receptors respond to only one taste quality and yet are simple enough to study in detail by present day anatomical techniques? We believe that in the blowfly, Phormia regina, we have found a system which responds primarily to only one taste quality which is simple enough to be studied anatomically and biochemically and which is similar enough anatomically to the mammalian system that information gained through study of its taste responses will enable us to generalize to higher organisms. The gustatory system of this fly consists of five cells, only two of which are involved with taste. One cell responds electrophysiologically and behaviorally only to sugars of various types, the other in identifiable manner to all other taste stimuli. In this sense this system can be identified as responsive to one taste quality provided the appropriate stimuli are applied and responses measured. The gross anatomy of this system consists of a hair cell with a pore on one end and two large fibers, within a chitinous sheath, which lead to the five

cells noted above. Only rudimentary electron microscope pictures of the anatomy of this system had been previously carried out. We have carried out preliminary work in this insect and have shown that there are, in the one cell which presumably responds to sugar, paired kinetosomes which can be traced by means of their rootlets to the fibers which enter the chitinous sheath. Each fiber is made of two structures, one set larger, the other, smaller. Each set or unit is filled with microtubules. These microtubules run the length of the sheath and end at the pore surrounded by granules which initially appear to resemble those seen at the pore of the human taste bud. This system appears to offer much promise for future anatomical and biochemical study.

Physiology. 1. Saliva. Salivary volume and composition, as well as taste acuity, has been studied in rats in whom all major salivary glands had been excised and who were treated with pilocarpine. Analysis of saliva has not been carried out in detail. Taste acuity, as noted by others, is markedly impaired. Salivary composition has also been studied in patients with cystic fibrosis of the pancreas. These studies confirm our earlier observations that the zinc concentration in the saliva of these patients was excessively high in comparison with normal subjects but that copper concentration was within normal limits. This finding may aid in the diagnosis of this condition due to the ease with which saliva can be obtained and metals measured, even in infants. Saliva has also been collected in patients with various taste abnormalities and is being evaluated with respect to its metal concentration.

2. Metals. Metal metabolism in some manner appears to be involved with the control of taste acuity in man and animals. In order to specify some aspects of this interaction it is necessary to know whether or not metal ions are present in the taste bud or the taste bud bearing papilla and whether or not this is a general or specific phenomena. This problem has been approached from three directions. First, whole tongues and valate papillae from normal rats were excised and either immediately frozen or fixed in glutaraldehyde and studied for their metal concentration by laser microprobe mass spectrometer techniques. Serial anatomical analysis of the whole tongue revealed that zinc was found only in the area defined by the valate papilla coincident with the finding of barium and strontium. Cross sectional analysis of the valate papilla itself revealed zinc to be present only in the epithelial layer of the papilla, that

layer containing taste buds and other sensory receptors, while none was found in the muscularis or adventitial layers. Similar analysis of circumvallate papillae was carried out in man, in patients with normal taste acuity and in patients with idiopathic hypogeusia. Zinc, barium and strontium was present in papillae from patients with normal taste acuity while zinc could not be clearly demonstrated in papillae from one patient with idiopathic hypogeusia. Technical problems with this technique severely limit interpretation of these results due to the inadequate resolution of the instrument used (about 25 microns). However, the results suggest that zinc is present in the taste bud bearing papillae in amounts adequate to measure but not in surrounding portions of the tongue and that zinc could not be measured by the techniques used in the papillae of one patient with idiopathic hypogeusia. Taste thresholds of this patient returned to normal after treatment with zinc ion.

Secondly, rat tongue and valate papillae were studied by electron microprobe microscopy but the results could not be interpreted due to technical difficulties with sample preparation.

Thirdly, Zn^{65}, 10 μc, were injected intravenously into rats and the tracer located in various tissues at various time periods following injection. With the exception of bone and kidney, no other tissue contained as much Zn^{65} per gm wet weight of tissue as did the tongue. Gross fractionation into posterior portion, containing the valate papilla, and the anterior portion, containing only fungiform papillae, was consistent with finding more Zn^{65} in the posterior rather than in the anterior portion of the tongue. These studies suggest that zinc may play a role in the papilla bearing taste buds.

3. Thiol-containing drugs and amino acids and their relationship to taste acuity. We have previously suggested that thiols are important as inhibitors in the taste process in man. To demonstrate this suggestion experimentally rats were fed diets with added D-penicillamine, cysteine or methionine, and their taste acuity was measured by the twenty-four hour, two bottle, free choice technique. These studies demonstrated that D-penicillamine produced decreases in taste acuity of great magnitude and significant differences in preference for NaCl between normal and D-penicillamine fed rats could be observed during presentation of solutions of NaCl as aversive as 0.75 M. Similar results were also shown for cysteine, a diet containing 2 gm cysteine per

100 gm mixed feed (Purina chow and dextrose) being more effective in reducing taste acuity than a diet containing 1 gm cysteine per 100 gm mixed feed. Methionine also reduced taste acuity but not to the degree noted with cysteine or D-penicillamine. Studies of copper and zinc metabolism in the rats fed cysteine demonstrated that no depletion of copper or zinc had occurred in blood or urine during the study which suggests that the effect on taste acuity was due to feeding the amino acid. The dose-response relationship suggests that the effect on taste can be increased by feeding more of this amino acid.

4. Taste Modifiers and Enhancers.

a. Miracle-fruit protein(MFP). The glycoprotein of the miracle-fruit berry has been known to alter the taste of sour substances to sweet. Its amino acid and sugar composition has been studied and some disagreement about its composition has been indicated although its molecular weight of 44,000 is well documented.

We have investigated the biochemical and physiological characteristics of the berry. Initially a new purification technique was developed which differs significantly from that of others. Berries, pulp and skin, are stirred several hours at 0-4°C in a suspension of 1/5 part insoluble polyvinyl pyrrolidone (PVP) in 10 parts 0.1 M $NaCO_3$ buffer, pH 10.5. The green-brown homogenate is filtered in the cold. The supernatant contains the active principle as determined physiologically by bioassay. A typical yield is 40 mg of TCA-precipitable nitrogen per 100 g wet weight of berry. The supernatant material from A is made 0.1 M in ε amino caproic acid and insoluble PVP is added. Over an one-half hour period glacial HAc is added dropwise to the stirred suspension at 0-4° C to lower the pH to 6.0-6.5. A solution containing the active principle is recovered therefrom by filtration. Typical yield is 20 mg of TCA-precipitable nitrogen per 100 gm wet weight of berry. The active principle is then adsorbed onto a short column of BioGel-CM, equilibrated with 0.1 M $NaPO_4$ buffer, pH 6.0, at 0-4° C. The column is then washed with 0.1 M buffer, pH 6.5, then the active principle is eluted stepwise with 0.1 M Na_2PO_4. Typical yield is 2 mg of TCA-precipitable nitrogen per 100 gm wet berry weight. The active principle, called MFP (miracle fruit protein) is adjusted to pH 6.0-6.5 and chromatographed on a column of carboxymethyl polyacrylamide gel. A shallow pH gradient in $NaPO_4$ buffer elutes the MFP over the pH range 7.0-7.3. Typical yield is 2 mg TCA-precipitable nitrogen per 100 gm

wet weight of berry.

Steps A,B and C removed condensed tannins which otherwise interfere with the stability of the MFP. Commercial preparations are unstable with respect to this factor and do not store well. A papain-like protease was also removed by these steps and this also reduced the yield of MFP reported by other investigators and contributed to the instability of the product. By this process, MFP recovery is at least two-fold greater than yields reported by other investigators and the scheme is significantly simpler. Elution diagrams of the step D chromatography reveal MFP peaks which vary from preparation to preparation. MFP is easily aggregated so that these fractions may represent various aggregates or compositionally different proteins. These potential differences are currently under investigation.

Gel filtration data have confirmed the apparent molecular weight of MFP as 44,000, as noted by other investigators. Gel filtration in a dissociating solvent, however, indicates that this value is a monomeric molecular weight. Disc electrophoresis studies are in progress as are studies on amino acid and carbohydrate composition. Limited study of primary sequence is contemplated, as are experiments aimed at removing carbohydrate components by enzymatic and chemical means. Effects of MFP on normal subjects have been carried out and indicate that the effects of our preparation are approximately 5 to 10 times, mg for mg, those of other investigators for altering the taste of sour to sweet. Utilization of MFP in patients with aglycogeusia demonstrate that there is no effect suggesting that an intact system for the sweet taste quality is required.

b. Protease activity. Anatomical and physiological studies carried out in the past year suggest that the taste receptors of the bud are exposed to the oral environment. We have also suggested that the membrane of the Type I cells are these taste receptors and that these are the receptors exposed to the oral environment. If these hypotheses are correct then placement of proteases into the oral environment should alter taste acuity in the direction predicted; i.e., disruption of secondary or tertiary structure of the protein of the receptor membranes with resulting interference with the pre-neural events of taste. Similarly each taste quality should be affected and the detection/recognition ratio should not be altered, each threshold increasing proportionately. These hypotheses were confirmed in experiments carried out

in subjects with normal taste acuity. Several proteases were introduced into the oral cavity (pronase, trypsin, papain) and each produced an immediate reduction in taste acuity coincident with slight burning sensations in the areas of the mouth where taste buds are located. Pronase,a relatively non-specific protease, was more effective in lowering taste acuity than was either trypsin or papain. The pronase effect lasted as long as 18 hours for the taste of bitter. Administration of proteases resulted in the sudden onset of hypogeusia for all taste qualities. Return of taste acuity to normal was slow and followed a consistent pattern. Initially, sour thresholds returned to normal, followed by salt and sweet thresholds, and lastly, thresholds for bitter. Introduction of lipases or amylases into the oral cavity had no effect on taste acuity. Similarly introduction of chymotrypsin or pepsin into the oral cavity did not alter taste acuity. Initial histological examination of valate papillae of rats treated with pronase suggest no anatomical changes in the bud were produced by introduction of this enzyme. These studies indicate that a protein containing receptor, subserving four taste qualities, is exposed to the oral environment and subject to the influence of proteases.

c. Taste modifiers. Monosodium glutamate, various nucleotides and peptides appear to modify taste acuity in such a way that placement of them in food makes the food more "flavorful". Studies of the effects of these substances on taste acuity in man are non-existant. Drugs and amino acids which affect metal metabolism also affect taste acuity when given systemically. To evaluate these various substances we have studied the effects of various nucleotides, peptides, membrane and ATPase modifiers and metal chelators on taste acuity in normal subjects in a manner similar to that carried out with proteases. These studies are currently in progress. Initial results indicate that metal chelators alter taste acuity in a manner similar to that noted for proteases. Initial results also indicate that nucleotides which influence taste acuity appear to do so by inhibiting only one taste quality, that for bitter, leaving other taste qualities intact. This latter result could explain the extremely common use of these substances in Japan where diets consist prominently of soy products. These products which are intensely bitter, can be made to taste quite palatable through the utilization of nucleotides and may represent a specific alteration in one taste quality similar to that observed with the utilization of miracle

fruit berries by African natives to alter the sour quality of their wheat products.

5. Role of vitamin A. Nine prisoners, housed on the metabolic unit of the Department of Medicine, University of Iowa School of Medicine, have been taking a diet deficient in vitamin A for 1 1/2 years. Serial studies of their vitamin A metabolism have been made including serum levels of the vitamin and its carrying protein (retinol binding protein), clinical and histological changes of skin, and changes in visual function. Prior to the measurement of any changes in vitamin A metabolism taste and smell acuity was measured and biopsies of circumvallate papillae taken. Patients were then depleted of vitamin A and thresholds again measured and biopsies taken. Patients were subsequently repleted with vitamin A and thresholds were measured again and biopsies taken. These studies indicate that when depleted of vitamin A thresholds for each of four taste qualities were significantly elevated and they returned to normal following vitamin A repletion. Changes in the taste buds of these patients have been detailed above. These studies demonstrate that vitamin A is required for normal taste acuity although the mechanism for this is not known.

Pathology. 1. Idiopathic hypogeusia with dysgeusia, hyposmia and dysosmia. This new disease was described in detail. Although previously mentioned in this report of 1970 the number of patients who suffer with this abnormality was not fully appreciated. We have received requests for assistance for treatment from over 3,000 patients who appear to suffer with this illness. Etiologically, the disease generally occurs following an attack of influenza but may occur following surgical procedures or without known cause. Pathologically there is a characteristic lesion of the taste bud which accompanies those cases studied. Treatment of 52 patients in a single blind study with zinc sulfate, in doses of 25, 50 or 100 mg orally, daily, as zinc ion, has indicated that thresholds for four taste qualities return to normal in 25%-67% of the patients treated. This disease, although not usually life threatening can be severely discomforting. The dysgeusic and dysosmic components of the disease allow the sufferer little or no pleasure from the intake of food. We are at present studying the efficacy of treatment of this disease with zinc sulfate in a double-blind design which will entail the eventual treatment of 108 patients in a modified crossover, block design.

2. Sjögren's Syndrome. Described in this report in 1970 this study has been continued and expanded such that 30 patients with this abnormality have been carefully evaluated and their loss of taste acuity documented. Median detection and recognition thresholds for each taste quality except for sweet, which is at the upper limit of normal, were elevated above normal in these patients. Treatment with cytoxan or other agents designed to correct their underlying abnormality has not been successful in returning their taste acuity to normal except in patients in whom salivary flow has returned significantly. Treatment of a limited number of patients with zinc ion was also unsuccessful in either returning salivary flow or in returning taste acuity to or toward normal in any patient, although equivalent treatment in patients with idiopathic hypogeusia was associated with significant beneficial effect.

3. Wegener's Granulomatosis and Midline Granuloma. Described in this report in 1970 this study has been continued and expanded such that 15 patients with these abnormalities have been carefully evaluated and their loss of taste acuity documented. Treatment of patients with Wegener's granulomatosis with cytoxan and carbohydrate-active steroids which produced a remission of their disease also produced a spontaneous return of taste acuity to normal. Treatment of a small number of patients with zinc ion, independent of treatment with cytoxan or steroids, also produced a return of taste acuity to normal in each patient so treated. Treatment of patients with midline granuloma with X-irradiation which produced a remission of their disease also produced some return of taste acuity toward normal. Virulent and aggressive infections in the nasal area were not associated with loss of taste. These findings may be useful in the oftimes difficult diagnosis of these rare diseases. Similarly, the loss of taste appears to be highly correlated with a recurrence of these diseases. The mechanism by which these changes occur is not known.

4. Type III familial dysautonomia. Two patients with diabetes mellitus, optic atrophy, neurogenic bladder, and neurosensory hearing loss with hyposmia, hypogeusia, hyperalanineuria, abnormal heat intolerance and other autonomic dysfunctions were studied in two sibs, aged 16 and 18.

The diabetes was insulin dependent, but resistant to ketosis. Plasma growth hormone and cortisol were normal, but neither responded to intravenous piromen. Arginine produced markedly

elevated and prolonged increases in immunoreactive plasma growth hormone. Metapyrone and ACTH produced normal responses. Oral papillae and taste buds were present. Nerve conduction velocities were normal. Both showed normal responses to intradermal histamine and pilocarpine, but abnormal cold pressor tests. Both had miosis after conjunctival placement of 2.5% methacholine. In a constant environment at 120°F with 12% relative humidity for 45 minutes their weight losses were significantly less than normal (80 gm), demonstrating the absence of sweating, while their body temperatures rose to 40°C with an abnormal temperature curve. Nevertheless, pilocarpine iontophoresis of the skin of the forearm produced normal sweat volumes. Abnormal glucose tolerance curves were demonstrated in the father and paternal uncle. The mother demonstrated miosis after conjunctival placement of 2.5% methacholine. Both parents showed hypogeusia and abnormal cold pressor tests but normal responses to intradermal pilocarpine.

These findings suggest that this syndrome belongs in the general category of familial dysautonomia. In contrast to Types I and II familial dysautonomia, this syndrome appears to have little peripheral component and to be due primarily to central autonomic dysfunction as opposed to the predominant peripheral autonomic dysfunction in Type II, and the central and peripheral autonomic dysfunction in Type I familial dysautonomia.

5. Aglycogeusia and Hypoglycogeusia. Inability to recognize the taste of any sweet substance, aglycogeusia was described in patients with congenital idiopathic hypoparathyroidism in this report in 1970. During this past year we have observed the presence of this defect and a partial defect, hypoglycogeusia, a quantitative decrease in the recognition of the taste of some sweet substances, in patients with Hand-Schuller-Christian disease, and in the mothers of these patients. This latter abnormality has also been observed in some unaffected siblings of the patients with this disease. These preliminary observations suggest that the ability to taste sweet may be controlled in some specific manner perhaps similar to that noted for the bitter taste of phenylthiocarbamide. These studies are also important in suggesting that a protein abnormality of an as yet unspecified type or location could be responsible for this defect and may offer us an important clue to the understanding of the basic mechanism of taste, particularly the taste of sweet.

6. Disseminated carcinoma of various types. Patients with carcinomatosis either untreated or treated with various toxic agents have developed anorexia and taste loss. These symptoms have not been susceptible to standard forms of therapy. We have been informed of patients with carcinoma of the pancreas, liver, stomach or lymphoma who have anorexia and loss of taste as their primary complaint subsequent to treatment of their underlying condition. Although the complaints of these patients are obviously complex and determined by many factors we have treated several of these patients with zinc sulfate in a single blind study to observe the effects of zinc ion on the anorexia and taste loss of these patients. These studies have been carried out through the cooperation of physicians at the City of Hope, Duarte, California and others throughout the U.S. Serum and urine were collected in metal free containers and zinc sulfate in the amount of 100 mg daily, as zinc ion has been administered. To date, studies on 5 patients have been completed. In two patients with carcinoma of the liver administration of zinc ion reversed the subjective complaints of anorexia and taste loss in both patients; in one patient with disseminated lymphoma change in appetite was evaluated subjectively while taste loss was evaluated objectively before, during and after treatment with zinc. Taste acuity in this latter patient returned to near normal levels and the anorexia disappeared during therapy. Each of these 3 patients had significant elevations of their serum zinc concentrations while taking the metal. Each has subsequently died of their underlying disease. One patient with carcinoma of the pancreas and one with carcinoma of the stomach were also studied. Their zinc levels in serum were normal prior to treatment and following treatment for one month there were no subjective changes in anorexia or taste acuity although serum zinc concentrations increased significantly. These studies suggest that the anorexia observed with carcinomatosis may be aided in some patients with the administration of zinc ion.

7. Hypertension and taste. Since 1906 the specific interrelationships between salt, fluid intake and hypertension have been well documented. Treatment of essential hypertension with salt and fluid restriction has been an accepted mode of therapy since that time. Many attempts have been made to link salt intake to hypertension, and while generally successful clinically, the correlation between the two phenomena has not been specifically convincing with respect to a model for possible mechanisms underlying hypertension. In an effort to investigate the possible role

of the appreciation of salt as an etiological factor in hypertension systematic studies in man and animals were undertaken. In man, thresholds for the taste of each of four qualities were investigated in patients with hypertension of several different etiologies. Untreated, with significant systolic and diastolic hypertension, or treated with drugs or surgery which lowered the blood pressure to the normal range, detection or recognition thresholds for the population of patients with hypertension studied did not differ from that of normal subjects. These results differed from those of other investigators who measured an aspect of taste acuity which is neither detection nor recognition of salt and found to be abnormal. The meaning of these latter relationships were not clear but this unresolved incongruity prompted us to measure the preference of a genetically hypertensive strain of rats (SHR) for NaCl in comparison with normal rats. These studies, briefly commented upon in this report in 1970, have been carried out in detail in SHRs using the twenty-four hour, two bottle, forced choice technique. SHRs show a significant decrease in their acuity for NaCl as indicated by their continued acceptance of solutions of NaCl normally rejected by other rats. This extends even to solutions as concentrated as 0.45 M NaCl. However, their preferences for other taste stimuli which are normally aversive such as quinine sulfate and HCl are not different from normal. Curiously, the intake of excessive quantities of NaCl in the SHR is not accompanied by excessive intake of fluid, something previously seen in each animal in previous experiments in whom hypogeusia occurred when produced by other means (e.g., feeding D-penicillamine, cysteine etc.). In order to document this finding we have repeated it on four occasions, each time with the same results. Given a choice between KCl and water or $NaHCO_3$ and water the differences between the SHRs and normal rats were not clear and these experiments will be repeated before any definitive interpretation will be made. Interpretation of the results of previous experiments also suggested that as the SHRs increased in age there was a decrease in their choice for NaCl over water such that the differences seen early in their life could be demonstrated but not as clearly as they were previously.

In an effort to clarify some of the variables in these experiments taste preference for various solutions were also studied in rats made hypertensive by the adrenal regeneration technique. Results of these experiments showed that taste preferences in rats made hypertensive by this technique did not

differ from those of control rats treated in a manner similar to the adrenal regeneration rats but without significant hypertension. Studies carried out by other investigators in rats made hypertensive by renal latex encapsulation or by deoxycorticosterone administration report that these animals show an aversion for NaCl and a polydipsia, exactly the opposite phenomena observed in the SHRs.

These conflicting data may allow the design of experiments which may elucidate the etiology of hypertension. Either the salt preference in SHRs may be related to their genetic differences from other rats or this preference which decreases positively with increasing age may be related to biochemical changes. This latter hypothesis raises the possibility that since renin production in the SHRs may decrease with increasing age there may be a correlation between these two events. Current studies are being undertaken to evaluate this possibility. Studies are also being undertaken to measure taste preference in the SHRs following correction of their hypertension with antihypertensive drugs and after treatment with various antiadrenergic, anticholinergic and antiserotinergic drugs.

Studies in patients with hypertension have taken a form similar to that described for rats. Patients are given a constant, dry 9 Meq NaCl diet and all fluid imbibed taken from either of two bottles, one containing distilled water, the other 150 mM NaCl. The subjects are given free access to these fluids over a 24 hour period. Total fluid imbibed, percent of NaCl imbibed and Na and K in the serum and urine are measured. Similar studies have also been carried out in two other groups of subjects; normal volunteers with normal blood pressure and without any family history of hypertension or cardiac disease and normal volunteers with normal blood pressure and significant family histories of hypertension or cardiac disease. Taste thresholds for 4 taste qualities are measured in each subject prior to the start of the test. Results of studies in patients with hypertension indicate that they prefer 20-60% of their daily fluid as 150 mM NaCl whereas normal volunteers without any family history of hypertension or cardiac disease imbibe less than 10% of their total daily fluid as 150 mM NaCl. These studies have lasted from 5 to 8 days. Preference for NaCl in normal volunteers with family histories of hypertension or cardiac disease differ from that of the other normal volunteers in that they usually begin taking in less than 10% NaCl but end the period of the

study imbibing more than 20% NaCl. These provocative preliminary results obtained from 5 patients with hypertension and 6 normal volunteers will be expanded during the next year by the continued study of normal subjects and patients with hypertension.

Pharmacology. Many drugs appear to affect taste acuity in an adverse manner. These drugs have been brought to our attention by reports made to medical journals throughout the world and sent along to our unit for comment. These drugs have caused subjective abnormalities of taste acuity in patients and were made known to the physician administering the drug by the patient's spontaneous complaints. In general, these reports have not been followed up systematically with quantitative testing of detection or recognition thresholds. These drugs include D-penicillamine, lincomycin, 5-mercaptopyridoxal, 6 axauridine triacetate, acetyl sulfosalicylic acid, griseofulvin, reserpine, ildamen, chlofibrate, valium and other tranquilizers and phenindione. Some of these drugs have similar actions and chemical structures and thereby may provide important clues to the mechanisms by which they act on taste acuity. For example, drugs which deplete the body stores of norepinephrine and which may alter taste acuity suggest that catecholamines may play some role in taste. Indeed, granules of at least two types are present in and around the taste bud and their composition is unknown. Similarly, the manner by which the taste information is transduced at the receptor in terms of a neurotransmitter agent is unknown. We therefore have undertaken a systematic evaluation of drugs which alter possible neurotransmitter agents in the rat in an effort to identify the manner by which this transmission may occur.

Education of physicians of taste abnormalities. Because of the prevalence of taste abnormalities in the population and the lack of knowledge of this subject by physicians who see patients with these abnormalities some method for supplying them with this information and techniques by which they can document and record these abnormalities must be made available. The technique used in our laboratory is cumbersome and time consuming. Therefore, a simplified modification of our technique has been devised and reduced to practice in the form of a Taste Testing Kit. With the aid of the Campbell Institute for Food Research this kit has been assembled and reduced to practice. Instructions for its use and Taste Record Cards by which results can be formally recorded have been made and will be available for distribution to interested physicians who wish to use this kit within the next 4 months.

As of this date over 500 physicians in the U.S. have requested to obtain such a kit.

Major Findings: Olfaction

Physiology. 1. Vitamin A metabolism. In the study previously noted with prisoners at the University of Iowa olfactory acuity for several vapors were measured before, during and after depletion of vitamin A by feeding a diet deficient in vitamin A and then readding vitamin A to the diet. Patients developed hyposmia while on the vitamin A deficient diet. This confirms the widely held belief that vitamin A plays some role in maintaining normal olfactory acuity. However, the role which vitamin A plays in olfaction is not clear. Studies in patients with acute viral hepatitis have demonstrated that olfactory acuity is impaired during the acute phase of the disease and it returns to normal as the disease process wanes. Attempts to correlate this hyposmia with changes in serum concentrations of vitamin A were unsuccessful. However, there was a significant inverse correlation between hyposmia and levels of bilirubin during the disease and a significant positive correlation between hyposmia and retinol binding protein (RBP), the major transport protein for vitamin A alcohol in serum. The correlation between vitamin A metabolism and olfaction suggests that a relationship between olfaction and vitamin A alcohol exists rather than between olfaction and vitamin A itself.

Attempts to correlate abnormalities of dark adaptation and hyposmia with vitamin A and RBP metabolism in patients with acute and chronic hepatitis were carried out in 12 patients studied at Harlem Hospital, New York City. Dark adaptation was normal in each patient although hyposmia and abnormalities of both serum vitamin A and RBP were present in most. The interrelationships between vision, olfaction and vitamin A metabolism, although provocative from the point of view of mechanism, are not yet clear.

2. Hypogonadism and olfaction. As noted in this report in 1970, a systematic evaluation of the effects of ablation of the olfactory bulb in female rats has been undertaken. These studies have been continued and appropriate control studies carried out. Ablation of the olfactory bulbs reduces the intake of food in female rats such that their body weights are significantly decreased from appropriate control animals. Paired feeding of rats

showed that starvation was a significant factor in the delay of sexual maturation but could not account for the profound delay in sexual maturation noted after ablation of the olfactory bulbs. FSH and LH measurements are at present being undertaken in these rats by Dr. Charles Barraclough, Department of Physiology, University of Maryland School of Medicine.

In man, the relationships between hypogonadism and olfaction has been carried out in a prospective manner. Using the data collected over the past 4 years in females with amenorrhea it is possible to predict, on a statistical basis, the association of Type II hyposmia with patients with gonadal dysgenesis and streak ovaries as opposed to the association of Type I hyposmia or anosmia with patients with hypogonadotrophic hypogonadism and unstimulated ovaries. Further studies are in progress at the present time.

3. Mechanism of hyposmia following laryngectomy. In this report in 1970 it was possible to state that bilateral surgical interruption of the 9th and 10th nerves was associated with the production of hyposmia as opposed to alterations in air flow which did not produce hyposmia. In patients in whom various surgical procedures involving the larynx have been carried out it has been possible to demonstrate that bilateral interruption of only the motor nerves to the larynx, i.e., the recurrent laryngeal nerves, was associated with hyposmia. The mechanism underlying this phenomenon is not clearly understood since sensory fibers involved in several reflex actions of the larynx accompany these motor nerves to the larynx. The feedback between nerves 9 and 10 and neural interconnections in the limbic cortex, as observed by Dell, may be ultimately responsible for this interaction. Negus' work, which demonstrates the role of larynx in olfaction in lower mammals and vertebrates, supports this entire concept on a behavioral level.

4. Nosology. The characterization of "primary" olfactory stimuli to correspond to the "primary" taste qualities of salt, sour, bitter and sweet has produced much confusion. In general, volatile, low molecular weight substances (<300) have an odor but their definitive grouping into qualities is not clear. In our studies of patients with aglycogeusia and with various forms of hyposmia it has been possible to isolate a specific "olfactory primary"; i.e., sweet. Patients with aglycogeusia can detect

gustatory and olfactory stimuli such as sucrose or chloroform, respectively, but cannot recognize them as sweet; indeed, all haloforms are detected by taste and smell but not recognized as sweet. This is in contrast to the ability of patients with anosmia who can taste all haloforms as sweet, but cannot detect or recognize the vapor. These studies demonstrate that sweet is a smell quality as well as a taste quality.

Pathology. 1. Sjögren's syndrome. Each of the 30 patients with Sjögren's syndrome studied demonstrated significant hyposmia of varying degree. As with taste acuity, no treatment schedule returned olfactory acuity to normal in any patient unless significant olfactory and nasal mucous was formed. Since these patients exhibit dry nasal mucous membranes due to the lack of nasal and olfactory mucous it is not surprising that olfactory acuity would diminish as suggested by the electrophysiological studies of Shibuya.

2. Wegener's granulomatosis and Midline granuloma. As noted previously patients with these two diseases suffer from hyposmia of varying degree. Of the 15 patients studied, those treated and in remission exhibited a return to or toward normal olfactory acuity. The mechanism underlying this change is unknown. However, in spite of the rarity of this disease, any patient with a history of hyposmia should be carefully evaluated to rule out either of these two diseases since early treatment can result in remission.

3. Idiopathic hyposmia. We have studied patients with idiopathic hyposmia over the past 4 years. They fall into two categories, congenital hyposmia, usually Type I, and acquired hyposmia, which can be either Type I or Type II. We have treated patients with both varieties with aqueous vitamin A, 50,000 units daily for up to three years. The hyposmia was returned to or toward normal in some patients with both congenital and acquired hyposmia but not in others. In an effort to document these changes we have undertaken a small double blind study in which a number of patients will be investigated, some receiving placebo for 6 months, some receiving aquasol A, 50,000 units daily, in an effort to evaluate the efficacy of vitamin A in the treatment of these ill defined conditions. Mr. M. Raff and Dr. W. Friedewald will assist us in the designing of this project.

4. Retinitis pigmentosa (RP). Patients with this abnormality represent a variety of retinal defects. In a single blind study, 22 patients with this abnormality have been studied over a period of 6 months. Documentation of their retinal defects were made independent of measurement of their taste, smell or auditory acuity. Results demonstrate that patients with RP fall into three categories of sensory abnormalities. The 10 patients studied with "typical RP" exhibited a demonstrable loss of taste and smell acuity. Auditory acuity was significantly decreased in approximately 45%. The 14 patients studied with "atypical RP", with pigmentary changes of the retina, exhibited some decreased taste acuity but normal olfactory acuity. Auditory acuity was also significantly decreased in about 40%. Only four patients with other retinal degenerations without pigmentary changes were studied. In general they exhibited hyposmia but normal taste acuity. None had decreases in auditory acuity. Too few patients in this latter category have been studied to consider them an adequate control group. At the present time measurements of vitamin A and RBP levels in serum do not clearly differentiate patients with RP. However, systematic studies of vitamin A and of RBP are at present underway to establish the presence or absence of any correlative biochemical changes.

These results suggest that patients with RP may have abnormalities of several sensory systems. Since RP is inherited in a dominant or an autosomal manner and since geneologies have been carefully documented by members of the NEI, patients with these abnormalities will be studied in a prospective manner such that the hypotheses made from results of the single blind study may be tested and evaluated. If these abnormalities do define patients with RP evaluation of receptor changes will be carried out.

5. The Molecular Basis of Olfaction. A report on the molecular basis of olfaction has been prepared for the Office of Naval Research during this past year. The purpose of the report was to review the state of the science of the sense of smell. In this report all major molecular theories of olfaction were evaluated and critically analyzed with respect to prevailing scientific thought and information. Over 5000 papers were reviewed, approximately 500 in detail and these were critically analyzed. Based upon these findings the requirements which an adequate molecular theory of olfaction must satisfy have been established.

Significance: Taste. We have operationally divided the taste system into 5 functional, component parts: (1) pre-neural events, (2) transduction events, (3) neural events, (4) CNS-feedback events on the bud and (5) humoral influences on the entire system. The major contribution which we have made is primarily limited to knowledge of the pre-neural events and to the definition of the transduction events.

We have clearly shown that there are at least two types of pre-neural events in taste. We have hypothesized that some form of chemical sieving controls the non-specific portion of the pre-neural events. Experiments carried out which demonstrate the inhibitory effects of thiol containing drugs and amino acids on taste support this concept. Similarly supportive are data demonstrating oral placement of proteases affect all taste qualities. These results suggest that proteases are effective on protein of the taste bud exposed to the oral environment. We suppose this protein is part of the membrane of the receptor cells of the taste bud. Our anatomical observations suggest that this is the Type I cell of the taste bud which comprise 80% of the cells of the bud.

We have also demonstrated that there is a specific portion of the pre-neural events of taste which relate to each taste quality. We have demonstrated specific pathophysiological abnormalities for the taste of sweet and also have isolated, in a purer form than have others, a substance which specifically alters the taste of sour to sweet. The specific taste events are most probably involved with the binding of tastant to the receptor membrane. Our anatomical investigations of the receptor of Phormia regina and of taste buds from patients with aglycogeusia have given us information of the anatomical receptor configuration related to appreciation of sweetness.

We have placed the entire problem of taste acuity within the framework of medical practice. The prevalence of the disease Idiopathic Hypogeusia which we have recently described indicates the need for an awareness and understanding of the loss of taste. The results of our single blind study indicates that zinc is beneficial in the treatment of patients with this disorder, although the mechanism is not yet known. With the discovery of this disease we have also demonstrated the first known pathology of the taste bud and we have published the first high power electron microscopic pictures of the normal histology and pathology

of the human taste bud.

We have also devised a test system by which a model for the role of salt intake in hypertension may be possible in rat and in man.

Olfaction. We have limited the role of vitamin A in olfaction to one which involves RBP and vitamin A alcohol. We have separated the abnormalities of vision from olfaction in hepatitis and this may lead to a clearer understanding of the role which vitamin A alcohol plays in olfaction. We have also clarified the interrelationship between olfactory function and gonadal function in lower mammals. We have identified sweet as a primary olfactory stimulus.

Proposed Course of Project: 1. To clearly establish whether or not zinc is efficacious in the treatment of patients with idiopathic hypogeusia through the completion of a double blind study of 108 patients with this disease.

2. To establish whether or not there is an etiological role for taste acuity for sodium in essential hypertension in man or in SHRs.

3. To clearly identify the anatomical characteristics of taste buds in fungiform, circumvallate and palatal papillae in man and in vallate papillae in rat.

4. To specify the anatomical abnormalities of taste buds in patients with aglycogeusia and otpathological abnormalities of taste.

5. To identify anatomically and physiologically the taste receptor in Phormia regina and to perform initial biochemical studies of specific binding of radioactive sugars to the receptor.

6. To define the interrelationship between RP and taste and smell abnormalities. If these do exist we will undertake an anatomical analysis of the taste receptor in patients with typical and atypical RP.

7. To complete the distribution of Taste Testing Kits to those physicians in the U.S. who have requested them.

Honors and Awards: None

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Patents

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Biochemistry

Endocrinology

Chemical

Kidney &

ANNUAL REPORT OF THE
LABORATORY OF KIDNEY AND ELECTROLYTE METABOLISM
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

The Laboratory of Kidney and Electrolyte Metabolism is studying the mechanism of water and electrolyte transport in the following tissues: mammalian nephron, amphibian bladder, and the avian erythrocyte. In addition experiments directed at elucidating the rate-controlling steps in cardiac contractility and the cause of a hereditary cardiomyopathy in hamsters also are in progress. This report will be concerned with the kidney, toad bladder and cardiac muscle studies. Detailed summaries of the erythrocyte experiments are included in the appended individual reports.

Mammalian nephron:

A method for the perfusion of isolated segments of the rabbit nephron in vitro was developed in this laboratory several years ago. This method permits direct measurements of transport under rigidly controlled conditions across the individual barriers of those epithelial cells responsible for the maintenance of water and electrolyte balance in the living animal. The in vitro preparation has a number of advantages over current in vivo techniques. Difficulties of interpretation of data attendant upon effects of the surrounding interstitial space, unstirred layers, inadequate control of the composition of the extracellular bathing fluid, etc. are avoided. The technique has proved useful in elucidating the mechanism of action of antidiuretic hormone and its intercellular mediator, cyclic 3',5'-monophosphate in the cortical collecting tubule of the rabbit. In addition it has been demonstrated that the path of bulk water flow along an osmotic gradient in this tissue is both through and between the cells. Antidiuretic hormone increases water permeability in the nephron in a manner analogous to that in amphibian structures such as frog skin and toad bladder. In the latter tissues the hormone increases net sodium transport, an effect presumed to be secondary to a change in the passive permeability of a limiting cell membrane to sodium, rather than a direct stimulation of the transport pump. It has not been established that sodium transport is accelerated by ADH in the kidney. Results based on in vivo studies in the rat and other mammals are conflicting. We have noted that the hormone increases the potential difference across the cortical collecting tubule without altering transtubular electrical resistance. Although the results may indicate that vasopressin accelerates net Na transport in the kidney by a direct effect on the sodium pump, the conclusion cannot be accepted as definitive without concurrent measurements of sodium flux. These are at present in progress. In preliminary studies employing ^{22}Na as a marker the unidirectional flux of Na from lumen to bath rose 10-20% following addition of 25 μU/ml of ADH to the bathing medium. Current studies are directed at measuring backflux, i.e., from the bathing medium to lumen under these circumstances to substantiate the tentative conclusions alluded to above.

Active transport of potassium from bath to lumen in exchange for reabsorbed sodium occurs in the cortical collecting tubule. It is probable, though not established, that the individual ions are transported through rather than between the cells. The mass of transported ions contained in the cells in the

course of transport is defined as the "transport pool." From its size, turnover rate and specific activity, the sites of ion transport and the transport properties of the individual cell membranes can be defined definitively. A method previously developed in this laboratory for the estimate of cellular pools of paraminohippurate and glucose in the proximal tubule has been adapted for the cortical collecting tubule to measure the K^+ transport pool. Cellular radioactivity is measured following addition of an appropriate isotope to either side of the epithelium. In order to identify the fraction of the intracellular radioactive K^+ contained in the transport pool as opposed to that in other non-transport pools in the tissue, the tubule is first perfused with a potassium-free solution. After the rate of net K secretion reaches a steady state, isotope is added to the bathing fluid. The tubule fluid is then sampled at frequent intervals until specific activity equilibrium obtains. The tubule may be removed during the equilibration period at specified times and the specific activity of the cells compared to the simultaneous specific activity of the secreted fluid in the lumen. If the specific activity of cell and luminal fluid potassium is identical, all of the cellular potassium must be resident in a transport pool. If not, other nontransport pools may be present. These studies are currently in progress as well as others which involve examination of the effect of a variety of diuretic agents on the transport processes in this portion of the nephron. With respect to the latter, it has been established that acetazolamide, ethacrynic acid and amiloride all alter the potential difference and electrical resistance and/or the sodium flux in this tissue.

The proximal tubule of the mammalian nephron reabsorbs 70-80% of the glomerular filtrate under normal circumstances. This process is obviously of considerable importance in the maintenance of hemostasis. Its mechanism is the subject of intensive investigation in laboratories in this country and abroad. It had generally been assumed that sodium is actively reabsorbed from the lumen creating a favorable electrical gradient for the passive movement of attendant anion, primarily chloride, out of the tubule fluid. A resultant increase in osmotic pressure in an inaccessible third compartment either between or closely adjacent to the blood surface of the epithelial cells is purported to provide sufficient driving force for reabsorption of water. The isolated proximal nephron transports an isosmotic salt solution from lumen to bath at a rate similar to that reported in in vivo studies. The sodium concentration of luminal fluid and that of the bulk bathing solution is unchanged during this process as it is in vivo. Furthermore, as in the intact nephron, interference with net water reabsorption by introduction of a poorly absorbable osmotically active solute (raffinose) lowers luminal sodium concentration to a limiting value approximately 30 mEq/L lower than its concentration in the bathing solution, without changing the osmolality of either solution. Though these results are consistent with the view that the primary driving force for the reabsorptive process is active transport of sodium, this has been questioned on the basis of in vivo results elsewhere. Crucial to an understanding of the process are precise estimates of the electrochemical driving forces for the individual ions, the individual conductivities and the permeability of the cell membrane to water. Since no measurable chemical gradient for sodium exists across the tubule wall under normal circumstances active sodium transport coupled with passive chloride reabsorption requires an appropriately oriented electrical gradient across the tissue. Initially a potential

difference (20 mV lumen negative) was reported in vivo. In recent years, however, this has been questioned and the observed potential attributed to artifacts in part attendant upon incorrect positioning of the exploring electrode in the tissue in vivo. The isolated tubule afforded a unique opportunity to re-examine the question since it is unnecessary to introduce the exploring electrode into the lumen by puncturing the epithelial wall. In contrast to the in vivo studies the electrode may either be positioned directly in the lumen through the open end of the tubule or placed in an appropriate bridge in direct communication with lumen fluid. Furthermore virtually total electrical insulation of both ends of the tubule has been achieved by a technique developed in this laboratory which employs a liquid dielectric, Sylgard 184, applied to the tubule ends. Using this technique it has been established that a potential difference of approximately 4.5 millivolts (lumen negative) is established during sodium chloride transport, unequivocal evidence of active sodium transport. Although the electrical gradient on the basis of preliminary calculations and studies appears sufficient to account for passive coupled chloride absorption further experiments are in progress to establish this with certainty. These involve direct measurements of chloride flux, potential difference and conductance under appropriate circumstances as well as transepithelial resistance.

Estimates of transepithelial resistance are also of considerable importance in determining the anatomical pathway for electrolyte movement across the tissue, that is, whether it occurs through or between the cells as is considered by many. The latter path would be the case were the transepithelial resistance higher than the sum of the individual transmembrane resistances (i.e. the transcellular resistance). Measurements of resistance are extremely difficult in the intact nephron and interpretation of the results requires multiple assumptions concerning the geometry of the tissue, the properties of the individual barriers, all of which are subject to considerable uncertainty. Many of these problems are obviated in the in vitro preparation and thus far it has been established that the transepithelial resistance is extremely low, consistent with the high permeability of the tubule to sodium and chloride. Methods for measuring longitudinal resistance of the epithelial cell as well as transcellular resistance of the serosal membrane are presently being developed.

Toad Bladder:

The toad bladder is a particularly useful model for examining the effects of hormones and other agents on water and electrolyte transport across oriented epithelial cells. It has been established in this laboratory that vasopressin induces an increase in water permeability and sodium transport which involves the intermediacy of the intracellular nucleotide, cyclic-AMP. The mechanism of action of cyclic-AMP on the processes of water and sodium transport is unknown. Since the nucleotide has recently been shown to stimulate the activity of a kinase which catalyzes the transfer of a labelled phosphate from ATP to an appropriate protein substrate in a number of tissues including toad bladder, it is likely that the isolation and characterization of a membrane protein, the phosphorylation of which is influenced by cyclic-AMP and/or hormone will afford important information with respect to the mechanism of action of the hormone on water and electrolyte transport. Thus far we have

confirmed that the toad bladder does in fact contain a cyclic-AMP sensitive protein kinase which catalyzes the phosphorylation of histone but not of protamine or phosvitin. No effect on the activity of the kinase by anti-diuretic hormone has been observed, however. We are currently developing methods for the isolation of toad bladder membrane fractions, separation of phosphorylated constituents within the membrane, in preparation for the studies outlined above. In addition a method for the estimation of the intracellular concentration of cyclic-AMP based on the binding of the nucleotide to a muscle kinase, a method developed in the Laboratory of Biochemical Genetics, has been successfully adapted for use in the toad bladder.

The current studies differ from earlier attempts in this laboratory and elsewhere in which intact tissue had been employed in an attempt to elucidate the mechanism of action of hormones. Pure epithelial cells devoid of surrounding interstitial tissue and muscle, both of which complicate interpretation of data, are now used. The cells are scraped from intact tissue previously incubated in collagenase for a required period of time. These cells respond metabolically to vasopressin, aldosterone and other agents as does the intact tissue. Thus ADH, for example, increases the oxidation of ^{14}C labelled glucose and pyruvate in both preparations, evidence of the viability of the tissue cells and their potential for experimental manipulations. The preparation thus far has permitted a clear definition of the mechanism of action of vasopressin, aldosterone, ouabain, an inhibitor of sodium transport, and amiloride, a diuretic agent, on sodium transport in the toad bladder. Cells and/or intact tissue are incubated with one of these agents under conditions in which net sodium transport is altered. At appropriate intervals pure epithelial cell sheets are removed and analyzed for their sodium content. In order to define the rate limiting step in transport affected by the agent the following simplifying assumptions have been made: the toad bladder epithelial cell is considered to be a three compartment system; a mucosal solution from which sodium is transported into the cell, a cell transport pool of sodium, and a serosal solution into which sodium is transported. Changes in the size of the transport pool within the cell coincident with alterations in transport rate may be interpreted in terms of a primary effect on sodium movement from mucosal solution to cell or from cell to serosal solution. Thus if sodium transport is accelerated by a hormone as a result of stimulation of a specific sodium pump on the blood surface of the cell this will be associated with a decrease in the sodium content of the tissue. Conversely, were transport to be accelerated primarily by increasing the entry of sodium across the mucosal permeability barrier, cell sodium content should increase. Analogous studies employing the intact tissue in this laboratory and elsewhere have repeatedly been negative, presumably a consequence of complications introduced by heterogeneity of the tissue. In the present study, however, in which pure epithelial cells were employed and analyzed, both aldosterone and vasopressin significantly increased the sodium content of the tissue, indicating that they must have induced a change in the permeability of the mucosal barrier which facilitated entry of Na into the cell. Ouabain, on the other hand, which decreases sodium transport in many tissues by interfering with the putative sodium pump resulted in an expected rise in cell sodium content. Finally, amiloride, a diuretic agent which decreases sodium reabsorption in the intact kidney and lowers net sodium transport in the toad bladder, induced a significant fall in the sodium content

clearly indicative of an effect of the agent on the permeability of the luminal surface to sodium rather than to inhibition of sodium pump. Additional studies directed at measuring changes in high energy phosphate donors and acceptors in the cell were also performed. Although neither aldosterone nor vasopressin altered the concentration of ATP in the epithelial cells at a time when sodium transport was accelerated, both agents resulted in a significant decrease in the concentration of creatine phosphate and a rise in the concentration of creatine. It has been concluded that acceleration of sodium transport is the primary event and the changes in metabolism secondary, rather than the converse as certain other investigators have suggested.

Other studies in this laboratory have defined the defect in cardiac muscle function in hereditary cardiomyopathy of hamsters. These animals, though devoid of evidence of heart disease at birth ultimately develop fatal congestive heart failure characterized by peripheral edema and pulmonary and hepatic congestion. Cardiac contractility in normal hamsters as in many other species is dependent upon two complementary phenomena. The first, the Bowditch phenomenon, is characterized by an augmentation in contractility at high rates of stimulation (or heart rate); the other, the Woodworth, by augmented contractility at low rates. Right ventricular muscle from hearts of hamsters in frank failure do not possess the Bowditch phenomenon whereas that from normal animals or those hamsters with the trait prior to development of failure do. The absence of the Bowditch phenomenon is a consequence of a disease-induced increase in the uptake of calcium from the bathing medium.

Serial No. NHLI-113
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of salt and water transport by proximal renal tubules.

Previous Serial No.: NHLI-168 and NHLI-169

Principal Investigators: Maurice B. Burg, M.D.
Jack Orloff, M.D.
Michael Lutz, M.D.
Dennis Waring, Ph.D.
Michael Horster, M.D.
Jean Cardinal, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: We devised a method for dissecting individual renal tubules from rabbit kidneys, keeping them alive in vitro, and measuring their function. This is an important advance over previous methods in renal physiology (such as clearance and micropuncture) since it permits the study of transport mechanisms in a way not previously possible. At present we are using this method to investigate the mechanism of salt and water transport in the proximal renal tubule, a problem which had not been elucidated by previous techniques despite considerable effort.

In our initial studies we found that the isolated perfused proximal convoluted tubules continued to absorb fluid from the lumen at an apparently normal rate, when bathed in rabbit serum and perfused with an ultrafiltrate of the serum (Burg, M.B. and Orloff, J., Amer. J. Physiol. 47:2016, 1968). Na concentration in the tubule fluid did not change during reabsorption under these conditions, indicating isotonic transport, but did decrease (by approximately 20%) when raffinose was included in the perfusate to limit water absorption. The transport mechanism was not able to lower Na concentration further because of the high Na permeability (10^{-4} cm sec^{-1} from isotope flux measurements) and correspondingly low NaCl reflection coefficient (σ_{NaCl} = .67) (Kokko, J., Burg, M.B., and Orloff, J., J. Clin. Invest. 50:69, 1971).

The major ions reabsorbed from the proximal tubule lumen are Na and Cl. In order to determine whether they are actively transported it is necessary to measure the electrical P.D. The results given above concerning Na concentration and permeability are in general agreement with those of micropuncture. This is not true, however, of the measurement of P.D. Previous measurements of electrical P.D. across the proximal tubule by micropuncture were conflicting. Values of both -20 and zero mV were reported by different workers, and the conclusion regarding ion transport mechanism depended on which number was chosen. Across isolated perfused rabbit proximal tubules we measured the electrical P.D. to be -4 mV (lumen negative) (Burg, M.B., and Orloff, J., Amer. J. Physiol. 219:1714, 1970). When active ion transport was eliminated by addition of ouabain the P.D. fell promptly to zero.

Considering that the P.D. is negative in the lumen and that Na reabsorption can take place both against a concentration and electrical gradient, Na transport must be active. Cl transport could be passive, driven by the negative luminal P.D. Evidence concerning the mechanism of chloride transport in proximal renal tubules, however, is also conflicting. Various investigators have considered that chloride transport may involve (1) passive reabsorption, (2) active reabsorption by a neutral pump mechanism together with Na, and/or (3) active secretion as HCl. Preliminary evidence from our laboratory favors (1), but the further studies listed below are required for proof.

These new experimental techniques are also being used to investigate the following additional problems:

1. The pathway for salt transport through the proximal tubule epithelium. At present the low electrical resistance and high salt permeability of the tubule are generally attributed to shunting of ions between the cells, although the supporting evidence is meager (a preliminary report in Necturus tubules several years ago). We intend to determine the pathways in rabbit proximal tubules by measuring transtubular and transcellular electrical resistances.

2. Cell communications. It seems likely that the contents of adjacent tubule cells may be in direct content through communications between the cells (gap junctions). This will be tested directly by measuring the longitudinal electrical resistance of the tubule wall.

3. The mechanism of action of diuretic drugs. It has been difficult, using standard micropuncture and clearance methods, to determine the mechanism of action of the various diuretics on

specific segments of the kidney tubule. Indeed, it is still unclear on which tubule segments some of the diuretics act. The direct and controlled study of individual tubule segments by in vitro perfusion should help answer these questions. Electrical methods have been chosen for initial screening since they provide information conveniently and rapidly as evidenced by their successful application to other epithelial tissues. Ouabain, the one drug previously tested, caused a rapid depolarization of the P.D. in proximal tubules. The agents to be tested on the proximal tubule are acetazolamide, theophylline, cyclic-AMP, ethacrynic acid, furosemide, amiloride, mersalyl and hydrochlorothiazide.

4. Permeability to macromolecules. It has been reported that relatively large molecules (MW > 500) permeate the proximal tubules of rats and Necturi. We wish to see if this occurs in isolated perfused rabbit proximal tubules, and if so, to define the transport mechanism. This question is important since simple passive permeability to such large molecules requires much larger openings through the epithelium than had been previously envisioned and would require re-evaluation of our concepts of the structure of the epithelium. Permeability to macromoles (e.g. proteins, dextrans, and other polysacchrides) will be measured from radioactive isotope flux or relative osmotic efficiency (reflection coefficient of Staveman).

5. Effect of protein on electrolyte transport in proximal tubules. Peritubular protein concentration is purported to be a major factor controlling the rate of electrolyte transport in proximal tubules, and has been reported (by others) to affect the rate of fluid absorption in isolated perfused rabbit proximal convoluted tubules. We wish to confirm the latter observation and to determine the mechanism involved. The effect of protein in the bath on electrolyte transport will be determined by removing protein from serum (ultrafiltration), replacing protein with other colloids, or adding protein to a high concentration.

6. Measurement of electrochemical potential difference in kidney tubules. Evaluation of the mechanism of ion transport in epithelia requires precise measurement of both the flows (net ion and water movement) and external forces (electrochemical P.D.'s). When identical solutions bathe the two sides of the epithelium and there is no hydrostatic pressure difference, the external force is in general equal to the electrical P.D. and is readily measured. It is usually desirable, however, to vary the flows by using non-identical solutions on the two sides of the epithelium, which complicates the measurement of electrochemical P.D. Under these conditions the electrical potential across the epithelium cannot be evaluated without either eliminating or measuring the liquid junction P.D.'s between non-identical solutions which are in contact. The liquid junction P.D. can be more or less eliminated by the use of bridges filled with concentrated KCl, and

the residual uncertainty of a few millivolts is unimportant in such tissues as nerve and muscle where the transmembrane P.D. and resistance are high. In the proximal kidney tubule, however, where the measured P.D.'s are only a few mV this system is unsatisfactory. The objective of the study is to develop satisfactory methods for evaluating electrochemical P.D. across proximal kidney tubules when non-identical solutions are used.

In order to resolve this problem we are attempting to evaluate the liquid junction potentials more accurately. A more promising approach, however, is to avoid the problem of liquid junction potential entirely by the use of the equivalent of the physical chemists' "cells without transference." This involves the preparation of microelectrodes reversible to the specific ions studied and the direct reading of the electrochemical P.D. between two such electrodes, one in the tubule lumen and one in the bath. Such electrodes reversible to Cl and to K have been built and tested by others using liquid ion exchangers. We are constructing similar electrodes for use in isolated tubules.

Methods: The major methodological advances have been in electrical measurements. Electrical methods are a powerful tool for study of ion transport and widely used in muscle and nerve physiology and for studying such epithelia as frog skin and toad bladder. They have been relatively little used in kidney physiology, however, because of the difficulty of applying them in micropuncture. When we discovered that Sylgard 184 liquid dielectric will electrically seal the ends of fragments of kidney tubules, detailed electrical study of isolated kidney tubules became possible. The initial studies were of electrical P.D. (Burg, M.B., et al Amer. J. Physiol. 215:788, 1968 and Burg, M.B. and Orloff, J. Amer. J. Physiol. 219:1714, 1970). Electrical resistance was first measured in the cortical collecting tubule (Helman, S., et al Amer. J. Physiol., in press). Measurement of transepithelial electrical resistance in proximal tubules had been a more difficult problem because of their fragility and because their low resistance and short space constant made it necessary to isolate extremely short lengths (approximately 200μ). In the new method which we have developed the electrical resistance is measured via the perfusion pipet in the tubule lumen, passing direct current through a platinum coating on the surface of the pipet and recording P.D. through the lumen of the pipet. Sylgard 184 is used to insulate the ends of the short length of tubule. The P.D. is also measured at the other end of the tubule. The resistance is calculated using cable theory, from the increment in electrical P.D. at the ends of the tubule associated with the electrical current.

In order to determine electrically whether there are ion shunts between the cells it is necessary also to measure the

transcellular resistance in order to see if this is higher than the transepithelial resistance, as it would be if there is an electrical leak between the cells. Transcellular P.D. and resistance can be measured using a single micropipet inserted into a cell from its luminal surface. This pipet is advanced into the tubule lumen by mounting it coaxially within the perfusion pipet. In principle P.D. and resistance can be measured simultaneously by using a bridge circuit to pass current through the single pipet. In a preliminary study a stable transcellular P.D. (approximately -50 mV) was found using this method, which indicates its feasibility.

Longitudinal resistance of the tubule wall was determined in non-perfused tubules. Direct current was passed through a segment of tubule immersed in either Sylgard 184 (liquid dielectric for electrical insulation) or serum and the resistance measured using a bridge circuit.

Major Findings:

1. Transepithelial resistance in the proximal tubule was approximately 7 ohm cm^2, a low value consistent with the high permeability of the tubule. Changes in NaCl concentration in the bath (replacement with non-electrolyte) caused a proportionate change in transepithelial conductance, indicating that much or all of the electrical current is carried through the tubule wall by Na and Cl.

2. The relative conductance of Cl vs. Na (i.e. its transport or transference number across the membrane) was determined potentiometrically. When NaCl concentration in the lumen was lowered by replacement with raffinose, the diffusion potential which developed was small, indicating approximately equal Na and Cl conductance.

3. The absolute Cl conductance (Cl transport number divided by total electrical resistance) is sufficiently large so that the Cl absorption can be accounted for by passive transport along the measured electrical gradient (-4 mV). However, the data are not sufficiently precise to rule out some active Cl transport. In order to investigate this possibility chloride transport will be measured while varying the chloride concentration difference across the tubule such that both secretion and absorption occur in different experiments. From the measured chloride electrochemical P.D. under these conditions it will be possible to calculate an independent value for chloride conductance and also to determine more precisely whether there is active chloride transport or not.

4. Longitudinal resistance of the tubule epithelium of

tubules immersed in Sylgard was approximately 10^8 ohm cm^{-1} and was identical whether or not the tubule lumen was filled with Sylgard indicating that the current was passing through the tissue itself. When the ends of the tubule were in Sylgard and a short central segment (approximately 100μ) was in serum (which was electrically grounded) there was no transmission of the P.D. from one Sylgard enclosed end to the other through the portion immersed in serum. This indicates a relatively low electrical resistance at the boundaries of the epithelium, possibly corresponding to the low transepithelial resistance. Further studies, including measurement of transcellular resistance, are required to distinguish whether the low boundary resistance is in the cell membranes or between cells.

Proposed Course of Project: Listed above in reference to the different areas studied.

Honors and Awards: None

Publications: Burg, M.B. and Orloff, J. Electrical potential difference across proximal convoluted tubules. Am. J. Physiol. 219:1714-1716, 1970.

Kokko, J.P., Burg, M.B. and Orloff, J. Characteristics of NaCl and water transport in the renal proximal tubule. J. Clin. Invest. 50:69-76, 1971.

Serial No. NHLI-114
1. Kidney & Electrolyte Metabolism
2. Renal Mechanisms
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of ion transport in renal cortical collecting tubules.

Previous Serial No.: NHI-192 and NHI-193 (Annual Report dated July 1, 1968 through June 30, 1969)

Principal Investigators: Maurice B. Burg, M.D.
Jack Orloff, M.D.
Gustavo Frindt, M.D.
Larry Stoner, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives:

1. The technique of perfusion of isolated renal tubules in vitro, which we developed, has proved especially useful for elucidating the mechanism of action of the antidiuretic hormone (ADH) in rabbit cortical collecting tubules (Grantham et al, Am. J. Physiol. 211:255, 1966; Ganote, et al, J. Cell Biol. 36: 355, 1968; Grantham, J.J. and Orloff, J., J. Clin. Invest. 47:1154, 1968; Grantham et al, J. Cell Biol. 41:562, 1969). Most of these studies were concerned with the mechanism by which ADH increases water permeability in the kidney tissue. ADH also has a second effect in frog skin and toad bladder, namely it increases Na transport. Whether it has a similar effect in kidney tubules is uncertain. Conflicting results have been reported in clearance studies. In the single micropuncture study reported, ADH increased Na permeability in the papillary collecting duct, but had had no effect on Na transport per se.

Previous results in this laboratory indicated that ADH transiently increased electrical P.D. in cortical collecting tubules (Burg et al, Am. J. Physiol. 215:788, 1968) without any change in electrical resistance (Helman, et al, Am. J. Physiol., in press). This is consistent with an increase of active Na transport, but provides only indirect evidence. One objective of the present project is to test directly the effect of vasopressin on Na transport in the cortical collecting tubule.

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2. We previously measured Na and K transport in cortical collecting tubules and established that Na is absorbed from the lumen and K secreted into the lumen, both by an active transport process (Grantham et al, J. Clin. Invest. 48:1915, 1970). These ions are most likely transported through the tubule cells. The mass of transported ion transiently contained in the cells in the course of transport is the "transport pool." From the size, turnover rate, and specific activity of the pool the cellular sites of ion transport can be identified, the transport properties of the individual cell membranes defined, and the effects of drugs and hormones on the latter elucidated. We are now attempting to measure ion transport pools, beginning with the K transport pool in cortical collecting tubule cells.

3. We are also studying the effect of various diuretic drugs on ion transport in this segment, since the site and mechanism of their action was previously poorly understood.

Methods: ADH effect: electrical P.D. was measured in order to detect whether added vasopressin was having an effect. Na transport was estimated from the unidirectional flux of ^{22}Na from lumen to bath. Albumin-^{125}I flux from lumen to bath was also measured, in order to detect damaged tubules with non-specific leaks.

Transport pools: The method previously developed in this laboratory for measurement of cellular transport pools of p-aminohippurate (Tune, B.M. and Burg, M.B., Am. J. Physiol. 217: 1057, 1969), and glucose (Tune, B.M. and Burg, M.B., Am. J. Physiol., submitted for publication) in proximal tubules will be adapted to study ions in collecting tubules. In this method cellular radioactivity (e.g. ^{42}K) is measured following addition of radioisotopes of the transported substances to either side of the epithelium. The principal problem, initially, is to identify what fraction of the ^{42}K radioactivity is contained in the transport pool, as opposed to possible other non-transport pools in the tissue. In order to identify the transport pool the tubule is perfused with a K-free solution. After a steady state of K secretion into the lumen is reached, ^{42}K is added to the bath and tubule fluid sampled at 1-2 minute intervals, ing the increase of K specific activity. The tubule is removed during the transient and specific activity of K in the cells compared to simultaneous luminal K specific activity. If the specific activity of cell and tubule fluid K is identical, all of the cell K is in the transport pool. If not, other non-transport K pools are present.

Major Findings:

1. Na isotope flux from lumen to bath and K isotope flux

from bath to lumen are low (approximately 500 x 10^{-12} M cm^{-2} sec^{-1}) consistent with the low rates of net Na and K transport previously measured and the high electrical resistance of this tissue.

2. In most experiments addition of ADH (25 μU/ml) caused a small (approximately 10 to 20%) increase in sodium flux from lumen to bath, consistent with an increase in active Na transport. We are presently measuring the effect of ADH on the back flux of Na (bath to lumen) in order to substantiate this conclusion.

3. Acetazolomide, ethacrynic acid and amiloride, the first drugs tested in collecting tubules, all caused changes in P.D., electrical resistance, and/or Na flux, indicating that it will be possible to determine the mechanism of their action in this tissue.

4. When ^{42}K was added to the bath during the steady state of K secretion, there was a gradual increase in specific activity of K in the lumen over several minutes. The delay in achievement of specific activity equilibrium is due to mixing with the cell K transport pool and indicates the feasibility of measuring the transport pool with this technique.

Proposed Course of Project:

1. To measure Na as well as K transport pool in this and other tubule segments.

2. To pretreat rabbits with high and low Na and K diets and with DOCA prior to study in order to investigate the phenomenon of "adaptation" to K (i.e. why more of a K load is excreted by the kidney when K intake is high) and the mechanism of action of the adrenal mineralocorticoids on the renal tubule.

3. To voltage clamp collecting tubules at different P.D.'s in order to determine the relationship between P.D. and Na and K transport.

Honors and Awards: None

Publications:

Grantham, J.J., Maurice B. Burg and Jack Orloff. The nature of transtubular Na and K transport in isolated rabbit renal collecting tubules. J. Clin. Invest. 49:1815-1826, 1970.

Helman, S.I., J. J. Grantham, and M. B. Burg. Effect of vasopressin on electrical resistance of renal cortical collecting tubules. Am. J. Physiol. (In press).

Serial No. NHLI-115
1. Lab. Kidney & Elec. Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A study of the mechanism of stimulation of sodium transport by vasopressin and by aldosterone by determining their effect on the electrolyte content and energy metabolism of the epithelial cells of the toad urinary bladder

Previous Serial Number: None

Principal Investigators: Joseph Handler, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Vasopressin and aldosterone increase the rate of active sodium transport by the toad urinary bladder. Experimental studies intended to define the rate limiting step in transport that is accelerated by each hormone have usually made the reasonable and simplifying assumption that the toad bladder epithelial cells can be considered as a three compartment system, a mucosal solution from which sodium is transported, a cell transport pool of sodium, and a serosal solution into which sodium is transported. Changes in the size of the cell transport pool coincident with changes in the transport rate can be interpreted in terms of an effect on sodium movement from mucosal solution to cell or from cell to serosal solution. Although numerous experiments have been performed, none has been successful. In large part, earlier experimental design and interpretation have been handicapped by the heterogeneous nature of the toad bladder, which consists of a single layer of transporting cells supported on a much thicker layer of connective tissue, blood vessels and smooth muscle bundles. In this study, a technique for the preparation of epithelial cells free of supporting tissue is developed and used to identify the cell surface at which the rate limiting step affected by aldosterone and vasopressin is located. In addition, the transporting cells are examined for an effect of the hormones on the concentration of certain high energy compounds in order to clarify the relationship between metabolic energy utilization and sodium transport.

Methods Employed: In paired experiments epithelial cells are scraped off intact collagenase treated bladders and incubated with no addition (control) or with vasopressin or aldosterone or an inhibitor of transport, amiloride, or ouabain. The viability of the cells and their responsiveness to the hormones or inhibitors is verified by measuring the rate of oxidation

of ^{14}C labelled glucose or pyruvate. Cells are collected for measurement of electrolytes by centrifuging the cells at a time corresponding to a steady state stimulation or inhibition of sodium transport. ^{14}C-inulin is used to estimate extracellular water. Cells are collected for measurement of labile high energy compounds by freezing in liquid nitrogen. The compounds are extracted into perchloric acid and assayed using highly sensitive fluorometric techniques.

Major Findings: The oxidative metabolism of the epithelial cells is affected by vasopressin, aldosterone, amiloride, and ouabain as expected from the effects of these agents on sodium transport and on the oxidative metabolism of the intact bladder. Vasopressin and aldosterone, at a time when they stimulate sodium transport, each increase the sodium content of the cells. At a time when they inhibit sodium transport, ouabain causes an increase in the sodium content of the cells and amiloride a decrease. These results are interpreted as indicating that each hormone stimulates sodium transport by increasing the rate at which sodium enters the cell from the mucosal solution. Ouabain inhibits the movement of sodium from the cell to the serosal solution, and amiloride inhibits the movement of sodium from the mucosal solution into the cell. Aldosterone and vasopressin do not change the ATP level in cells significantly, but each hormone causes a marked fall in the concentration of phosphocreatine. This effect is interpreted as indicating that each hormone primarily stimulates sodium transport, the increase in metabolism occurring secondarily.

Proposed Course of Project: Project is completed.

Honors and Awards: None

Publications: None

Serial No. NHLI-116
1. Lab. Kidney & Elec. Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A study of the effect of vasopressin and certain other hormones and drugs on the concentration of adenosine 3',5'-monophosphate in the epithelial cells of the toad urinary bladder

Previous Serial Number:

Principal Investigators: Jeffrey Stoff, M.D.
Joseph Handler, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: A considerable amount of evidence has been gathered in this laboratory indicating that an early step in the action of vasopressin on mammalian kidney and toad urinary bladder is stimulation of the enzyme adenyl cyclase, resulting in increased production of adenosine 3',5'-monophosphate (cyclic-AMP) and that the ensuing accumulation of cyclic-AMP within the cell elicits the permeability and transport changes characteristic of the effect of the hormone. In previous studies the concentration of cyclic-AMP in the toad urinary bladder was measured in the intact bladder which is a heterogeneous tissue composed of smooth muscle, blood vessels, and mesothelial cells, as well as the epithelial cells that determine permeability and respond to vasopressin. In this study, the epithelial cells will be isolated and their content of cyclic-AMP measured, utilizing a new and sensitive technique. In addition, the effect of a number of other hormones and certain drugs which modify the response to vasopressin will be examined for their effect on the cyclic-AMP content of the epithelial cells.

Methods Employed: Preliminary experiments indicate that reproducible results are obtained by incubating the intact bladder with vasopressin and then rapidly scraping off the mucosal epithelial cells with the edge of a glass microscope slide. The cells are quickly frozen in liquid nitrogen and extracted in trichloracetic acid containing tracer amounts of tritium labelled cyclic-AMP for measurement of recovery which is usually 60 to 75%. The trichloroacetic acid is removed by cation exchange chromatography and the samples lyophilized and taken up in a small volume of water. Cyclic-AMP is measured using the method of Gilman, which depends upon displacement of radioactive cyclic-AMP from high affinity binding sites on an enzyme isolated

from beef muscle. The specificity and sensitivity of the assay are suitable for the purposes of this study, permitting accurate measurement of picogram quantities of cyclic-AMP.

Major Findings: Depending upon conditions prior to separation, the epithelial cells contain 5 to 20 picomoles per milligram of protein (about 2×10^{-6} moles per liter of cell water). Fifteen minutes of incubation with vasopressin causes an increase of approximately 50% over the concentration in paired control tissue.

Proposed Course of Project: The time course for the effect of vasopressin will be established as well as a concentration that elicits a reproducible submaximal response. Experiments will then be designed to establish directly the effects on cell cyclic-AMP levels of such agents as prostaglandins, catecholamines, adrenal steroid hormones, and chlorpropamide, which have been shown to alter the permeability and transport response to vasopressin.

Honors and Awards: None

Publications: None

Serial No. NHLI-117
1. Lab. Kidney & Elec. Metabolism
2. Electrolyte Transport
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A study of cyclic-AMP dependent protein kinase in the toad urinary bladder

Previous Serial Number: None

Principal Investigators: Rodney Omachi, M.D.
Joseph Handler, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Previous work in this laboratory has established the thesis that vasopressin alters the permeability and transport properties of the toad urinary bladder by stimulation of the production, and thus the accumulation within the responsive cells of adenosine 3',5'-monophosphate (cyclic-AMP). Recent reports have described the presence in many animal tissues of a cyclic-AMP dependent enzyme, a protein kinase that catalyzes the phosphorylation of certain proteins, using the γ-phosphate of ATP as phosphate donor. In some tissues in which the phosphorylated protein is an enzyme, it has been shown that the activity of the enzyme is markedly altered by phosphorylation and that this change in activity can explain some of the physiological changes that are evoked in the tissue by the hormone. This study is intended to characterize cyclic-AMP dependent protein kinase activity in the toad urinary bladder so that its activity can be assayed after experimental manipulation of the bladder, and to identify the protein substrate(s) for the enzyme in the tissue. Presumably the protein substrate(s) for the enzyme has a key role in the permeability and transport response to vasopressin.

Methods Employed: Epithelial cells (the vasopressin sensitive portion of the toad urinary bladder) are scraped from collagenase treated bladders. After exposure to vasopressin the cells and paired control tissue are frozen and ground to a fine powder in liquid nitrogen, transferred to phosphate or Tris buffered extraction solution and gently homogenized with a glass homogenizer. Activity is assayed in various centrifugal fractions by incubation with P^{32}-γ-ATP, ± protein substrate, ± cyclic-AMP. The reaction is terminated by adding cold 8% trichloroacetic acid and the phosphorylated protein trapped and then washed on a millipore filter. P^{32} on the filter is determined with liquid scintillation counting.

Major Findings: Protein kinase activity is present in the 20,000 x G particulate fraction and the 20,000 x G and 100,000 x G supernatant fractions. Cyclic-AMP stimulates activity two to four times when histone is the substrate. There is little or no stimulation by cyclic-AMP when casein, protamine, or phosvitin are used as substrate, although these proteins are phosphorylated. Although cells incubated with vasopressin for 5 to 30 minutes are known to respond to the hormone, extracts from these cells have the same cyclic-AMP independent and dependent protein kinase activity as extracts from paired control cells. Aldosterone which enhances the response of the toad bladder to cyclic-AMP, is also without effect on cyclic-AMP dependent or independent protein kinase activity.

Proposed Course of Project: Recent reports indicate that the effect of cyclic-AMP on protein kinase activity is rapidly reversible. This may explain the similarity of the activity found in extracts prepared from vasopressin and control tissues, and this approach will not be pursued further. Cyclic-AMP dependent protein kinase of high specific activity will be prepared from toad bladder epithelial cells using techniques that have been successful in other tissues. Proteins and other materials from the epithelial cells that are phosphorylated by the enzyme in the presence of cyclic-AMP will be isolated and characterized.

Honors and Awards: None

Publications: None

Serial No. NHLI-118
1. Lab. Kidney & Elec. Metabolism
2. Membrane Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A study of the effect of prostaglandin E_1 and 7-oxa-13-prostynoic acid on the sodium transport and permeability properties of the toad urinary bladder

Previous Serial Number: None

Principal Investigators: William C. Albert, M.D.
Joseph S. Handler, M.D.
Jack Orloff, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The response of the urinary bladder of the toad to vasopressin is characterized by an increase in the rate of active sodium transport and by an increase in permeability to water. Vasopressin elicits this response by stimulating the formation and thus the intracellular accumulation of cyclic-AMP. Prostaglandins are naturally occurring fatty acids that in low concentrations have been shown to have a variety of effects in many tissues. Previous studies in this laboratory indicated that prostaglandin E_1 (PGE_1) inhibits the water permeability response of the toad bladder to vasopressin but not the water permeability response to cyclic-AMP, an effect interpreted as inhibition of the effect of vasopressin on adenyl cyclase, the enzyme that catalyzes the formation of cyclic-AMP. Other laboratories have confirmed these observations while observing no effect of PGE_1 on the sodium transport response to vasopressin. This would imply that there are two different vasopressin-responsive adenyl cyclase systems in the toad bladder, only one (that associated with the water permeability response) sensitive to the inhibitory effects of PGE_1. This study is intended to explore the effect of prostaglandins on the stimulation of sodium transport by the bladder and to test the effect of 7-oxa-13-prostynoic acid, a newly synthesized analog of prostaglandin that has been reported to be a competitive inhibitor of PGE_1 in the ovary and in smooth muscle.

Methods Employed: Sodium transport is measured by the short-circuit current technique and water permeability by measuring the rate of water flow from a dilute solution bathing the mucosal surface to Ringer's solution bathing the serosal surface of the bladder. Experiments are designed to use paired tissue from the same toad.

Major Findings: 10^{-6} M PGE_1 frequently stimulates sodium transport slightly. Despite this stimulatory effect, PGE_1 inhibits the submaximal stimulation of sodium transport elicited by low concentrations of vasopressin, and has no effect on comparable stimulation of sodium transport by cyclic-AMP. A similar pattern of inhibition of the submaximal water permeability response to vasopressin and no inhibition of the response to cyclic-AMP was again obtained. Thus, these studies do not support the suggestion that only the adenyl cyclase mediating the water permeability response to vasopressin is inhibited by PGE_1. 7-oxa-prostynoic acid inhibits sodium transport in the concentration (10^{-4}M) used by others to study its effects on intact tissues. 8×10^{-7} M 7-oxa-13-prostynoic acid, a concentration that does not alter the sodium transport rate, does not alter the inhibitory effect on the sodium transport or water permeability response to vasopressin of a 300-fold lower concentration of PGE_1, indicating that it is probably not a competitive inhibitor of PGE_1 in this preparation. The effect of higher concentrations of 7-oxa-13-prostynoic acid on sodium transport by the bladder raises the possibility that the inhibitory effect on the response to PGE_1 observed in other tissues is non-specific.

Proposed Course of Project: The effect of PGE_1 on the concentration of cyclic-AMP in the epithelial cells of the toad bladder will be measured as well as its effect on the changes in cell cyclic-AMP levels elicited by vasopressin.

Honors and Awards: None

Publications: None

Serial No. NHLI-119
1. Kidney & Electrolyte Metabolism
2. Electrolyte Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Volume regulation in duck erythrocytes

Previous Serial Number: NHLI-176

Principal Investigator: Floyd M. Kregenow , M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Previous studies in this laboratory have shown that duck erythrocytes are capable of returning to their original volume in a hypotonic medium after first swelling. This capability requires that duck erythrocytes possess a "volume controlling mechanism" that is sensitive to some cellular parameter associated with cell size. The previous annual report (1970) also indicated that duck erythrocytes can regulate their volume in hypertonic medium after first shrinking. Although this response had not been characterized completely, it was apparent that it also required a "volume controlling mechanism."

(I) The cellular changes and the nature of the membrane events associated with the volume adjustment in hypertonic media were first compared to those in hypotonic media. This analysis facilitated a comparison of the operation of the "volume controlling mechanism" in two separate environments during which markedly different changes in cellular content developed. The changes in cell volume in an isotonic medium, induced by the addition or removal of norepinephrine (See Annual Report, 1969), were then examined to determine whether a similar mechanism was operative here.

(II) This comparison involved the use of Ouabain, a cardiac glycoside, which specifically inhibits the Na-K exchange pump mechanism in mammalian erythrocytes and is known to inhibit the active transport of Na and K in duck erythrocytes. This agent was used to determine whether the "volume controlling mechanism" and the classical Na-K exchange pump mechanism were interrelated.

(III) A calculation of the quantity of electrolytes and H_2O lost during the volume regulatory phase (previously called the osmoregulatory phase) in hypotonic media supported the concept that the changes in cell size were the result of a nearly isosmotic adjustment in cell water. A similar calculation during the hypertonic volume regulatory phase, however, failed to support this concept as the quantity of electrolyte gained was in excess of

the quantity required for amount of water accumulated. A direct attempt was therefore made to establish whether the changes in cell size brought about by the "volume controlling mechanism" were examples of "isosmotic intracellular regulation". The activity of water in the intracellular and extracellular phase was compared before, during, and after the adjustments in cell size by noting the melting point of a microscopic aliquot of cells and medium.

(IV) Several cellular components were examined during the adjustments in cell volume to establish which cellular parameter was regulating cell size by controlling the activity of the "volume controlling mechanism."

Major Findings: IA. As reported previously (Annual Report, 1970) the response of duck erythrocytes in hypertonic media can be divided into two phases; an initial rapid phase of osmotic shrinkage and a more prolonged volume regulatory phase in which the cells swell until they reach a new steady state volume. The hormone, norepinephrine, need not be present in the bathing medium, as was originally thought, for the readjustment in cell volume to occur. Under optimal conditions, duck erythrocytes incubated in a hypertonic solution without norepinephrine and with 10^{-4} M propranolol, a potent inhibitor of the norepinephrine response, swell after first shrinking and approach their lower steady state, isotonic volume. Optimal conditions include an increase in the $[K]_o$ from a "normal" value of 2.5 mM/L to 15 mM/L and a hypertonic solution whose osmolality does not exceed the osmolality of an isosmotic solution by more than 110 mosmoles. In contrast there are no extracellular limitations to volume adjustment in hypotonic media other than a bathing medium so dilute that hemolysis occurs. Provided optimal conditions are present, an increase in the tonicity of the bathing medium initiates a response which is just sufficient to return the cell to its original volume. At a given tonicity the relationship between an increase in the extracellular potassium concentration and the increase in cell volume is a gradual one, increasing from a $[K]_o$ of 2.5 in mM/L to a value of 15 mM/L, where a maximal response is obtained. The increase in cell volume is always accompanied by a gain in KCl which is more than sufficient to explain the changes in cell volume if the cells remain in osmotic equilibrium with their environment. There is also a much smaller initial gain in Na content followed by a decrease but not to original levels. The changes in Na and K are initially associated with a 5-10-fold increase in Na and K influx and Na^{24} and K^{42} loss from the cells. The fluxes return toward normal as the cells swell and reach their original volume. The increase in Na permeability is largely ouabain insensitive as is the increase in K permeability. The generalized increase in permeability is associated with the active accumulation of potassium (the gain in potassium occurs against an electrochemical gradient). If duck red cells are incubated in a hypertonic solution under conditions where they remain shrunken ($[K]_o$ = 2.5 mM/L), the same generalized increase in permeability develops which, however, persists and is not associated with the active accumulation of potassium.

The marked similarity between the cellular and membrane changes reported here and those seen previously when cells are stimulated in an isotonic medium

by the hormone norepinephrine (See Annual Report, 1970) is interpreted as indicating that an identical "volume controlling mechanism" produces the latter response. This is taken as evidence that the "volume controlling mechanism" can also regulate cell size in an isotonic medium under appropriate circumstances. The similarities between the action of norepinephrine and hypertonicity suggest that both pertubations produce an identical cellular change (the norepinephrine effect is presumably mediated through cyclic-AMP) which in turn initiates the regulatory mechanism. An laternative explanation is that hypertonicity can regulate adenyl cyclase activity. The marked dissimilarities between the cellular and membrane changes during the hypertonic and hypotonic volume regulatory phase is interpreted as indicating the effector portion of the "volume controlling mechanism" is different in the two responses. No hypothesis can yet be presented to explain all of the changes during the hypertonic volume regulatory phase. There are numerous parallels however on the one hand, between "facilitated diffusion" of organic solutes and the translocation of potassium at a $[K]_o$ of 2.5 mM/L and on the other hand besides the obvious idfficulty of applying the "pump-leak" hypothesis to each.

IB. The response of enlarged duck erythrocytes (which had been incubated in an isotonic bathing medium with a $[K]_o$ of 15 mM/L and 10^{-6} M/L of norepinephrine) was followed once the factors responsible for the cell enlargement (an elevated $[K]_o$ and norepinephrine) were removed. Experimentally this was accomplished by washing norepinephrine treated enlarged cells in an ice-cold, norepinephrine-free isotonic solution ($[K]_o$ = 2.5 mM/L) and then incubating them for 90 minutes in a solution identical to the wash solution, but with 10^{-4} M Inderol. Cells treated in this fashion shrunk rapidly and approached the lower steady state isotonic volume within 90 minutes. This response is identical to that seen during the hypotonic volume regulatory phase and is comparable to the response of cells in a hypotonic solution of approximately 240 mosmoles. Potassium and chloride are the major electrolytes lost during the response. This loss is sufficient to explain the changes in cell size if the cells remain in osmotic equilibrium with their environment. The alteration in cellular K content is associated with a large transient increase in K efflux and either no or a slight change in K influx. Raising the $[K]_o$ of an isotonic solution to 107 mM/L prevents the cells from shrinking. Initially prevention is associated with an enormous increase in K influx and either no or a slight change in the already accentuated increase in K efflux.

The marked similarity between the cellular and membrane changes seen during the hypotonic volume regulatory phase and those reported here is interpreted as indicating that an identical "volume controlling mechanism" produces the latter response. This is taken as further evidence that the "volume controlling mechanism" can regulate cell size in an isotonic medium. It also supports the concept that the changes in cell size respond to differences in a cellular parameter rather than an extracellular factor. The changes in cell potassium and permeability reported here, like those that occur during the hypotonic volume regulatory phase can be explained best by postulating that there is a transient increase in the diffusional pathways for potassium.

II. Ouabain at a concentration of 10^{-3} or 10^{-4} does not significantly

alter the volume of control cells nor the changes in cell volume that develop during either the hypotonic (1) or hypertonic (2) volume regulatory phase or upon the addition (3) or removal (4) (See Section IA) of norepinephrine from an isotonic medium. During the 90-minute incubation period, during which these experiments were performed, ouabain does alter the Na and K content in each group of cells. In (1) and (4) the gain in Na (-6 mM) and loss of K (-6 mM) is not significantly different from that of control cells. The ouabain induced changes in the cation content of norepinephrine treated cells (3) has been reported previously (Annual Report, 1970). In a 435 mosmolar hypertonic solution (4) the cells gain approximately 12 mM Na and lose approximately 12 mM K.

These findings indicate that the "volume controlling mechanism" can operate independently of the classical Na and K exchange pump mechanism - at least in the sense that the changes in cell volume produced by the former are unimpaired when the latter is inhibited. The changes in the Na and K content of ouabain treated cells in (4) indicate that in this condition the Na and K exchange mechanism operates normally more rapid than usual to convert a gain in cell Na to one in potassium. In the presence of ouabain, the "volume controlling mechanism" simply utilizes NaCl instead of KCl as the major intracellular osmotic substance.

III. The melting point of a microscopic aliquot of medium and hemolyzed frozen-thawed cells was analyzed on a nanoliter osmometer. Within the error of the measurement (3-5 mosmoles), the osmolality of the medium and cells remained identical, before, during, and after the changes in cell size that develop in all four of the experimental conditions described in Section II.

These findings indicate that duck erythrocytes are at osmotic equilibrium with their environment normally and as they vary their volume in all four experimental conditions. Volume adjustments under these circumstances are therefore, by direct analysis, examples of "isosmotic intracellular regulation" and comparable to the kind of cellular adaption seen more commonly in invertebrate animal cells.

IV. Measurements of either Na, K and total Na and K content and concentration or cellular osmolality as the cells shrink or swell, indicate that in the four experimental conditions described in Section II, none of these factors could serve as the required cellular parameter. They either fail to vary or vary so much during the changes in cell size that they lack the necessary consistency required of this factor.

Significance to Biomedical Research and the Program of the Institute: Cell size is an intrinsic property of all animal cells. Since it has been established that most animal cells are at osmotic equilibrium with their environment and contain 70-90% water, the major portion of cell size is determined by factors which control the solute content of the cell. The extent to which a mechanism identical to the one in the duck erythrocyte controls the solute content and size of other cells will determine the major significance of the present work. If an identical mechanism is present in other cells, most disciplines in biology would have an overlapping interest in this mechanism.

Proposed Course of Project:

1. To establish the presence of the volume controlling mechanism in mammalian and non-mammalian erythrocyte ghost preparation.

2. To attempt to isolate the membrane elements responsible for volume regulation in the duck erythrocyte. This study would require the use of an electron microscope.

3. To examine the relationship between extracellular sodium and the cell swelling induced by a hypertonic solution or norepinephrine, especially as this relationship relates to amino acid transport.

Honors and Awards: None

Publications: Riddick, D. H., Kregenow, F. M. and Orloff, J.: The effect of norepinephrine and dibutyryl cyclic-AMP on cation transport in duck erythrocytes. J. Gen. Physiol. (In press).

Kregenow, Floyd M.: The response of duck erythrocytes to non-hemolytic hypo-osmotic media - evidence for a volume-controlling mechanism. J. Gen. Physiol. (Accepted for publication).

Kregenow, Floyd M.: The response of duck erythrocytes to hyperosmotic media - further evidence for a volume-controlling mechanism. J. Gen. Physiol. (Accepted for publication).

Serial No. NHLI-120
1. Lab. Kidney & Elec. Metabolism
2. Exp. Cardiovascular Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Immunochemical comparison of cardioglobulin and rat muscle E-C coupling complex

Previous Serial Number: NHLI-177

Principal Investigator: Stephen Hajdu, M.D.

Other Investigators: Edward J. Leonard, M.D.

Cooperating Units: None

Project Description:

Objectives: To determine whether there is any chemical relationship between rat cardioglobulin-C and rat skeletal muscle excitation-contraction (E-C) coupling complex.

Methods Employed: Isolated frog hearts were used for binding and bioassay of rat cardioglobulin-C. These hearts were also used as a specific immunoabsorbent for anti-cardioglobulin C antibody (see results). Standard techniques were used for antibody production, immune fluorescence microscopy and separation of cardioglobulin-C from other serum proteins.

Major Findings: A. Development of an immunochemical assay for rat cardioglobulin-C. A rat protein fraction containing cardioglobulin-C activity was injected with complete Freund's adjuvant into rabbits. The resulting antiserum had anti-cardioglobulin-C activity, as judged by binding to surface-membrane cardioglobulin-C sites of a BC frog heart, and inhibition of the biological activity of cardioglobulin-C. This antiserum makes 3 precipitin lines when reacted in gel with whole rat serum. After absorption by passage of antiserum through a series of BC frog hearts, one precipitin line, called precipitin line 3, is no longer detected. Our tentative conclusion, therefore, is that rat cardioglobulin-C is in precipitin line 3. Thus, we at least have an immunochemical marker for a protein which up until now we could identify only by its biological activity on the frog heart.

B. Immunochemical relationships between cardioglobulin-C and rat E-C complex. A purified fraction from rat muscle, containing the E-C complex, was set up in gel against anti-cardioglobulin antiserum. Two precipitin lines formed, which made lines of identity with rat cardioglobulin-C fraction antigens. One of these identity lines was precipitin line 3 which we believe represents cardioglobulin-C. Thus there appears to be a close

immunochemical relationship between cardioglobulin-C and a protein in the E-C coupling fraction. The evidence suggests that cardioglobulin-C and the muscle E-C complex are chemically similar.

Proposed Course of Project: Project completed.

Honors and Awards: None

Publications: None

Serial No. NHLI-121
1. Kidney & Electrolyte Metabolism
2. Experimental Cardiovascular Diseases
3. Bethesda, Maryland 20014

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of cardiac failure in hamsters with hereditary cardiomyopathy

Previous Serial No.: None

Principal Investigators: Christian J. Posner, M.D., Ph.D.
Stephen Hajdu, M.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The contractility of most mammalian cardiac muscle is the result of two independent phenomena whose contribution to overall contractility varies with the frequency of stimulation. At high frequencies it is determined by the Bowditch phenomenon (BP). Contractile tension over this frequency range is supported by a decrease in intracellular potassium which amplifies the effect of the constant amount of free extracellular calcium entering the fiber at every frequency of stimulation. Over the low frequency range, contractile tension is dependent on the Woodworth phenomenon (WP) which is mediated by calcium supplied from bound sources. The amount of this available calcium increases during the rest intervals. Since the alkaloid ryanodine eliminates only the WP it allows accurate separation of the contribution of WP and BP to overall contractility.

When it was reported that a strain of hamsters spontaneously develop a primary cardiac failure we were able to study the effect of the disease on these phenomena (BP and WP). If as the result of the disease both phenomena are equally depressed, it would have to be concluded that a defect in the contractile system is the common cause of the depression since it would be unlikely that the disease would affect two independent functions equally. On the other hand if the disease is restricted to only one of the phenomena, leaving the other intact, this would indicate a normal contractile system with the point of attack on the specific mechanism serving only the affected phenomenon.

Methods: The hamsters were divided into 3 groups. Group 1

contained healthy golden hamsters, group 2 cardiopathic hamsters without signs of cardiac failure, and group 3 cardiomyopathic hamsters with severe congestive heart failure. Isometric contractions were recorded from right ventricular muscle preparations suspended in Krebs solution vigorously gassed with a mixture of 95% 0_2 and 5% $C0_2$. For experiments involving measurement of ^{45}Ca uptake during the depolarized state, muscles were suspended in Krebs solution in which all sodium was replaced with potassium (Potassium - Krebs). The control muscle in each pair remained unstimulated and was suspended in normal Krebs solution. Each solution contained the same specific activity of ^{45}Ca (equivalent to 10^6 cpm ^{45}Ca/ml). At the end of the incubation period the muscles were washed in ice-cold Krebs solution every 4 minutes for 40 minutes. At the end of the washing period, the muscles were blotted dry, weighed on a torsion balance and homogenized in Krebs solution in a glass tissue grinder. Aliquots of the homogenate were dried on planchets and radioactivity counted in a low-background counter.

Major Findings: The interval-tension curve for the healthy golden hamster and that of the cardiomyopathic hamsters without evidence of cardiac failure is similar, with tension increasing at both high and low frequencies indicating a normal BP and WP. The interval-tension curve of the cardiomyopathic hamsters with severe congestive heart failure shows a parallel course with the curves obtained from the first two groups but only over the range of the WP (that is, between 2-60 sec.). At high frequencies when the BP begins to develop in the normal animals (2 sec. or less) the curve of the diseased animals continues to decline as though the BP were missing in these animals.

In order to determine whether the BP is absent in the diseased hearts, the hearts in all groups were treated with ryanodine. No tension was recorded in the WP range in all three groups but undiminished contractility was observed in the BP range in hearts from the controls. The hearts of animals suffering from congestive failure developed no tension over the entire frequency range showing that all tension exhibited prior to ryanodine treatment was produced by the WP. A normal BP is present in the hearts of the young cardiomyopathic animals free of the signs of cardiac incompetence but absent by the time that cardiac failure has reached its final stage.

The absence of BP could be the result of a malfunction in either step determining the BP, that is failure of the decrease in intracellular potassium with increasing frequency or failure of entry of free extracellular calcium into the fiber. The functional state of the potassium mechanism was evaluated by use of the cardiac glycosides. While addition of scilliroside produced a shift of the BP toward the lower frequency range in

healthy hamster hearts, it did not increase the tension over the high frequency range in the diseased hearts which would have indicated return of the BP. A large dose of glycoside eventually led to the development of contracture in the diseased heart indicating that there was no lack of response to this intervention which lowers intracellular potassium.

The probable explanation for the absence of increased contractility over the BP range in the cardiomyopathic hearts is therefore failure of extracellular calcium to enter during depolarization. This hypothesis was evaluated by measuring ^{45}Ca uptake of heart muscle with a polarized and depolarized membrane. Calcium uptake, expressed as cpm ^{45}Ca/mg muscle, was determined in five separate muscles for both normal and cardiomyopathic hamsters after incubation times of 20, 40 and 80 min.

In the healthy hamster hearts, the ^{45}Ca accumulation during the depolarized state continues to increase above that in the polarized state with increasing incubation times. In the cardiomyopathic hamster hearts there is no significant increase in ^{45}Ca accumulation during the depolarized state compared with that in the polarized state. Thus the change that takes place in these animals later in life and leads to a fatal cardiac failure is an impermeability of the membrane to entrance of free extracellular calcium during depolarization of the membrane.

Significance to Biomedical Research and the Program of the Institute: This represents the first analysis on the cellular level of the mechanism of a naturally occurring primary cardiac failure. This study shows that with progression of the disease, the cardiac muscle fails to allow entrance of free extracellular calcium during depolarization. As a consequence of this, the heart is deprived of normal contractility at the physiological heart rates. Due to this lack of calcium entrance during depolarization the cardiac glycosides also become ineffective and this is in good agreement with long standing clinical observation (cardiac failure refractory to glycosides). This study delineates the course of future research directed toward the correction of cardiac failure.

Proposed Course of Project: The correction of the defect leading to cardiac incompetence in the diseased hamsters can proceed in two directions:

1. Restoration of calcium entry: This approach has been explored by the use of epinephrine, the most potent known agent to increase permeability of calcium during the depolarized state. While epinephrine made some improvement in calcium entry in some of the animals, in others it had no effect. In our judgment this indicates that when the disease has reached its final state

there is no practical way to restore calcium entry. However prevention of the closing of the membrane to calcium entry would be correct in principle.

2. Improvement in the Woodworth phenomenon: Extension of the WP to cover the high frequency range, as in skeletal muscle, would restore contractility by increasing the amount of calcium released from bound sources. Our study has already shown that this mechanism of compensation is present to a small degree in the diseased hamster hearts but this is not adequate to maintain normal cardiac function. Enough evidence has been collected which suggests that the WP is based on the function of the cardioglobulin system. Improvement in the function of this system seems the most feasible procedure to restore contractility in the failing heart.

Honors and Awards: None

Publications: Hajdu, S.,and Posner, C. J.: Absence of Bowditch phenomenon in the ventricular muscle of hamsters with hereditary cardiomyopathy. Am. Heart J. 81: June, 1971 (In press).

Biochemistry

Chemical

Biochemical

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMICAL GENETICS
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

Three aspects of molecular biology were studied during the past year: Neurobiology, mechanisms of protein synthesis, and the biochemistry of rare constituents of tRNA.

A. NEUROBIOLOGY

1. Neuroblastoma

Clonal lines of neuroblastoma provide an unusual opportunity to explore steps in neuron differentiation as well as functional characteristics of mature neurons. The cells resemble neuroblast stem cells yet are capable of multiplying rapidly in vitro and give rise to cells exhibiting many properties characteristic of differentiated neurons. Many new clones of mouse neuroblastoma C1300 were obtained during the past year and were examined for enzymes catalyzing the synthesis of putative neurotransmitters. Three types of neuroblastoma clones with respect to transmitter synthesis were found: 1) Clones with no apparent transmitter; 2) adrenergic clones; 3) cholinergic clones. Two varieties of human neuroblastomas are known: adrenergic and non-adrenergic tumors. On the basis of studies with mouse neuroblastoma a third class of human tumors may be expected: i.e., tumors of cholinergic neuroblasts. A simple, highly sensitive assay for acetylcholine transferase was developed that provides a ready means of identifying such tumors.

It is possible that clonal differences in enzymes for neurohormone synthesis reflect genetic heterogeneity; alternatively, the differences may be a function of the developmental potential of the normal neuroblast precursor of the mouse neuroblastoma.

The formation of synapses between the various neuroblastoma clones and dissociated cells from cardiac and skeletal muscle was investigated. The rate of contraction of single cardiac cells or of colonies in vitro was determined by recording pulsations on video-tape and displaying the output on recorder paper, using a video-analogue output. Neuroblastoma cells were found to form a strong physical connection with muscle cells. Stimulation of neuroblastoma cells altered the rate of contraction of muscle cells. These and additional results suggest that neuroblastoma cells are capable of synapsing in vitro; however, further work is needed to substantiate this possibility.

2. Genes for neuronal properties expressed in neuroblastoma x L cell hybrids.

Mutant clones of mouse neuroblastoma were selected by mutagenesis and exposure to 6-thioguanine. The electrically excitable neuroblastoma cells were fused with electrically passive L cells having a hitherto undescribed electrical marker. Hybrid clones, examined 10-40 generations after fusion, were found to be electrically excitable. The results show that at least part

of the genetic information from neuron differentiation can be functionally expressed in neuroblastoma x L cell hybrids. No evidence for a pleiotropic repressor terminating neuron differentiation was found. In fact, most N x L cell hybrids were more active electrically than the parental neuroblastoma line. Somatic cell hybridization applied to normal neuroblasts should provide a relatively simple means of establishing clonal lines of cells derived from different types of neurons. The levels of acetylcholinesterase of N x L cell hybrid clones were determined 30-50 cell generations after cells were fused. The neuroblastoma parent and all N x L cell hybrids contained acetylcholinesterase; appreciable amounts of enzyme were not detected in the L cell parent or in L x L hybrids. The results show that neuroblastoma genes for acetylcholinesterase continue to be expressed after fusion.

Results obtained with neuroblastoma clearly show that rapidly dividing cells still retain the ability to express neuronal functions and that some neuronal genes remain active in somatic cell hybrids. The possibility that neurons may be capable of initiating a program of neuron differentiation in recipient cells also deserves consideration.

3. Acetylcholinesterase

We have previously shown that neuroblastoma cells contain acetylcholinesterase and both excitatory and inhibitory acetylcholine receptors and that these factors respond to regulatory mechanisms in vitro. Thus, purification and characterization of the factors were initiated to define the relationship between acetylcholinesterase and acetylcholine receptors and the nature of the regulatory mechanisms. Acetylcholinesterase was found to be associated with membranes; however, the enzyme now has been purified extensively and is now being characterized.

4. Cyclic AMP

A simple, rapid assay for adenosine 3',5'-cyclic monophosphate was developed by A. Gilman that is based upon competition between labeled and unlabeled cAMP to a protein, presumably a cAMP-dependent protein kinase. The nucleotide-protein complex is then adsorbed and washed on a cellulose ester filter. The assay is sensitive to 0.05-0.10 pmoles of cAMP, and is being used to explore cAMP metabolism in neuroblastoma and other cell types that are grown in vitro.

B. MECHANISMS OF PROTEIN SYNTHESIS

1. Mammalian peptide chain termination

Mammalian peptide chain termination has been studied in vitro using a modification of the formyl methionine (fMet) release assay developed for bacterial studies. Release of fMet from rabbit reticulocyte fMet $tRNA_f$·ribosome intermediates requires a protein release factor (R), terminator codon template, and GTP. Release factor has been purified to >95% homogeneity prepared from rabbit reticulocytes, and partially purified from guinea pig liver and Chinese hamster liver. The purified reticulocyte R factor has a

native molecular weight by G200 Sephadex chromatography of ≈ 255,000, is composed of subunits, has a ribosomal dependent GTPase activity, and is active in the fMet release assay using the templates UAAA, UAGA, or UGAA. Multiple release factors of differing codon specificity have not been found. The release reaction is inhibited by GDPCP which is consistent with the demonstrated GTPase activity being essential for peptide chain termination. The release reaction is also inhibited by sparsomycin, anisomycin and gougerotin, inhibitors of the mammalian peptidyl transferase. This extends the correlation found in bacterial systems implying peptidyl transferase participation in peptide chain termination. These antibiotics do not inhibit R factor ribosomal-dependent GTPase activity. More recently, we have studied the formation of R·radioactive terminator codon·ribosome intermediates. GDPCP and ethanol can be used to stabilize such intermediates for quantitation by Millipore filter retention. These studies suggest that recognition sites for all three terminator codons are part of a single large R factor.

C. BIOCHEMISTRY OF RARE CONSTITUENTS OF tRNA

1. The effect of methylated bases on the biological activity of Met-tRNA.

The properties of normal and methyl-poor Met-tRNA were compared in a variety of test systems. The results show the following: a) of the two normally occurring isoacceptors of Met-tRNA, the nonformylatable species is acylated more quickly than the formylatable species, and both of these are aminoacylated by purified methionyl-tRNA synthetase more rapidly than is methyl-poor Met-tRNA; b) on reverse-phase chromatography, methyl-poor Met-tRNA, Met-$tRNA_M$ and Met-$tRNA_F$ can all be separated from each other; c) methyl-poor Met-tRNA serves as a substrate for the transformylating enzyme, is recognized by initiation factors and is excluded from recognition by elongation factors in a manner indistinguishable from normally methylated Met-tRNA.

2. The involvement of leucine in tRNA modification.

When relaxed-control E. coli is deprived of methionine, new species of tRNA are formed. Since methionine is a source of methyl groups of tRNA, the newly formed tRNA is methyl-deficient. We have now found that new species of tRNA also accumulate when relaxed-control E. coli are starved of leucine. New RNA synthesis is necessary for the phenomenon since the appearance of new species is prevented by leucine starvation of stringent control cells or uracil starvation of relaxed-control cells. Leucine starvation leads to formation of new species that accept the amino acids leucine, arginine and histidine. This distribution suggests that leucine is involved in a modification reaction specific for those tRNA's that recognize codons beginning with C. This is in keeping with previous data from this and other laboratories showing that tRNA's recognizing codons beginning with U contain isopentenyl adenosine and those recognizing codons beginning with A contain N-(purin-6-ylcarbamoyl)-threonine. Further studies on these new species of tRNA will continue.

3. The biochemistry of N-(purin-6-ylcarbamoyl)-threonine in tRNA.

The most recently discovered minor constituent in tRNA is N-(purin-6-carbamoyl)-threonine (PCT). Since it has been found in the tRNA for the amino acids isoleucine, methionine, serine and lysine, it has been suggested that its occurrence will be restricted to those tRNA's whose codons begin with A. We have initiated a program to study the biosynthesis and distribution of PCT in tRNA. By exposure of a "relaxed control" threonine auxotroph of E. coli to threonine under conditions where RNA synthesis is allowed and protein synthesis is blocked, we have been able to specifically incorporate label from radioactive threonine into PCT. This is evidence that threonine is at least a partial precursor of PCT. Our plan for the immediate future is to utilize specifically labeled tRNA to deduce which species of tRNA contain PCT. This should allow us to test the distribution hypothesis mentioned above.

Serial No. NHLI-122
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Initiation of Mammalian Protein Synthesis

Previous Serial Number: NHLI-339

Principal Investigator: Daniel Twardzik

Other Investigator: Alan Peterkofsky

Cooperating Unit: Ettore Appella, National Cancer Institute

Project Description:

The project described last year is progressing as anticipated. Pyrrolidone carboxylic acid (PCA) occurs as the N-terminal amino acid of many mammalian proteins. Our experiments have been aimed at determining the precursor of this unique amino acid. Using cells derived from a mouse plasmacytoma, we have been able to incorporate label from either radioactive glutamic acid or glutamine into protein. Degradative analysis of labeled protein originated from labeled glutamic acid shows label in only PCA and glutamic acid. On the other hand, if label originated from glutamine, the labeled protein contains label in PCA, glutamic acid and glutamine. This finding indicates that PCA must be derived more directly from glutamic acid than from glutamine, a finding contrary to a suggestion in the literature. Tests of enzyme activities in cell extracts support our data: While glutamine synthetase is absent, glutaminase activity is substantial. This suggests that the mechanism whereby glutamine is converted to PCA involves a prior conversion to glutamic acid. These observations will be pursued with the intention of elucidating the mechanism of PCA formation from glutamic acid.

Honrs and Awards: None

Publications: None.

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Serial No. NHLI-123
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Aminoacyl-tRNA Synthetases

Previous Serial Number: NHLI-340

Principal Investigator: Takis Papas

Other Investigator: Alan Peterkofsky

Cooperating Units: None

Project Description:

The mechanism of several aminoacyl-tRNA synthetases has been studied in this and other laboratories. While each aminoacyl-tRNA synthetase shows specificity for its unique amino acid, the characteristics of the reactions involved are generally similar. There are, however, three notable exceptions to this similarity; the synthetases for arginine, glutamine and glutamic acid all require the cognate tRNA's for demonstration of the ATP-PPi exchange reaction, while all the other synthetases show no such tRNA requirement. We have initiated a study of the mechanism of these unique aminoacyl-tRNA synthetases with a view to exploring these unusual properties.

Honors and Awards: None

Publications: None.

Serial No. NHLI-124
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Neuroblastoma System as a Model for Neuron Differentiation.

Previous Serial Number: None

Principal Investigator: Marshall Nirenberg

Other Investigators: Takehiko Amano, Elliott Richelson and Edward J. Thompson.

Cooperating Units: None

Project Description:

Clonal lines of neuroblastoma provide an unusual opportunity to explore steps in neuron differentiation as well as functional characteristics of mature neurons. The cells resemble neuroblast stem cells yet are capable of multiplying rapidly in vitro and give rise to cells exhibiting many properties characteristic of differentiated neurons. Many new clones of mouse neuroblastoma C1300 were obtained during the past year and were examined for enzymes catalyzing the synthesis of putative neurotransmitters. Three types of neuroblastoma clones with respect to transmitter synthesis were found: 1) Clones with no apparent transmitter; 2) adrenergic clones; 3) cholinergic clones. Two varieties of human neuroblastomas are known--adrenergic and non-adrenergic tumors. On the basis of studies with mouse neuroblastoma a third class of human tumors may be expected: i.e., tumors of cholinergic neuroblasts. A simple, highly sensitive assay for acetylcholine transferase was developed that provides a ready means of identifying such tumors.

It is possible that clonal differences in enzymes for neurohormone synthesis reflect genetic heterogeneity; alternatively, the differences may be a function of the developmental potential of the normal neuroblast precursor of the mouse neuroblastoma.

The formation of synapses between the various neuroblastoma clones and dissociated cells from cardiac and skeletal muscle was investigated. The rate of contraction of single cardiac cells or of colonies in vitro was determined by recording pulsations on video-tape and displaying the output on recorder paper, using a video-analogue output. Neuroblastoma cells were found to form a strong physical connection with muscle cells. Stimulation of neuroblastoma cells altered the rate of contraction of muscle cells.

These and additional results suggest that neuroblastoma cells are capable of synapsing *in vitro*; however, further work is needed to substantiate this possibility.

Honors and Awards: None.

Publications:

Seeds, N. W., Gilman, A. G., Amano, T. and Nirenberg, M. W.: Regulation of axon formation by clonal lines of a neural tumor. *Proc. Natl. Acad. Sci.* 66: 160-167, 1970.

Serial No. NHLI-125
1. Biochemical Genetics
2. Medical Genetics
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mammalian Peptide Chain Termination

Previous Serial Number: None

Principal Investigator: Arthur Beaudet

Other Investigators: C. T. Caskey and J. L. Goldstein

Cooperating Units: None.

Project Description:

Mammalian peptide chain termination has been studied in vitro using a modification of the formyl methionine (fMet) release assay developed for bacterial studies. Release of fMet from rabbit reticulocyte fMet $tRNA_f$·ribosome intermediates requires a protein release factor (R), terminator codon template, and GTP. Release factor has been purified to >95% homogeneity prepared from rabbit reticulocytes, and partially purified from guinea pig liver and Chinese hamster liver. The purified reticulocyte R factor has a native molecular weight by G200 Sephadex chromatography of ≃ 255,000, is composed of subunits, has a ribosomal-dependent GTPase activity, and is active in the fMet release assay using the templates UAAA, UAGA, or UGAA. Multiple release factors of differing codon specificity have not been found. The release reaction is inhibited by GDPCP which is consistent with the demonstrated GTPase activity being essential for peptide chain termination. The release reaction is also inhibited by sparsomycin, anisomycin, and gougerotin, inhibitors of the mammalian peptidyl transferase. This extends the correlation found in bacterial systems implying peptidyl transferase participation in peptide chain termination. These antibiotics do not inhibit R factor ribosomal dependent GTPase activity. More recently we have studied the formation of R·radioactive terminator codon·ribosome intermediates. GDPCP and ethanol can be used to stabilize such intermediates for quantitation by Millipore filter retention. These studies suggest that recognition sites for all three terminator codon are part of a single large R factor.

Honors and Awards: None.

Publications:

Goldstein, J., Beaudet, A., and Caskey, C.T.: Peptide chain termination with mammalian release factor. Proc. Natl. Acad. Sci. U.S. 67: 99-106, 1970.

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Beaudet, A., and Caskey, C. T.: Mammalian peptide chain termination II. Codon specificity and GTPase activity of release factor. Proc. Natl. Acad. Sci. U.S. 68: 619-624, 1971.

Serial No. NHLI-126
1. Biochemical Genetics
2. Macromolecules
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biochemistry of Rare Constituents of tRNA

Previous Serial Number: NHLI-338

Principal Investigators: Maurille Fournier, Marcia Litwack, Jane Marmor, Donald Powers

Other Investigator: Alan Peterkofsky

Cooperating Units: Dr. Herbert Dickerman, Johns Hopkins University; B. P. Doctor, Walter Reed Army Institute of Research

Project Description:

A. Biological Studies of Isopentenyl Adenosine in Transfer RNA (Marcia Litwack and Alan Peterkofsky).

Isopentenyl adenosine (iPA) occurs only in those species of tRNA responding to codon whose first letters begin with U. Our previous studies showed that, in Lactobacillus acidophilus, mevalonic acid was the precursor of iPA. iPA content of tRNA can be varied dependent on the concentration of mevalonic acid in the culture fluids. Our studies have shown that, while the gram-negative organism Escherichia coli contains the thiomethylated derivative of iPA in its tRNA, the tRNA of the gram-positive Lactobacillus contains unmodified iPA, as does mammalian liver tRNA.

We have now completed our studies on a comparison of the biological properties of iPA-rich and iPA-poor tRNA. All attempts to show a requirement for iPA in tRNA for maximum rate or extent of aminoacylation or peptide-bond synthesis have been negative. We conclude, in conflict with some other data in the literature, that iPA is not necessary for the acceptance or transfer functions of tRNA in Lactobacillus.

B. The Importance of Methylated Bases for the Biological Activity of the Methionine tRNA (Jane Marmor and Alan Peterkofsky).

This project, initiated last year, has been brought to a conclusion. While the properties of methyl-deficient tRNA for several amino acids have been previously examined, extending the study to include the tRNA for methionine was felt to be warranted. Methionine tRNA is unique in several respects: It is a substrate for the enzyme that forms N-formyl-methionyl-tRNA, it specifically recognizes initiation factors, and it is the only tRNA that does not form a complex with elongation factors. We have compared the

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properties of normal and methyl-poor methionyl-tRNA in a variety of test systems and found the following: (a) of the two normally occurring iso-acceptors of met-tRNA, the non-formylatable species is acylated more quickly than the formylatable species and both of these are aminoacylated by purified methionyl-tRNA synthetase more rapidly than is methyl-poor met-tRNA; (b) on reverse-phase chromatography, methyl-poor met-tRNA, met-$tRNA_M$ and met-$tRNA_F$ can all be separated from each other; (c) methyl-poor met-tRNA serves as a substrate for the transformylating enzyme, is recognized by initiation factors and is excluded from recognition by elongation factors in a manner indistinguishable from normally methylated met-tRNA.

C. The Involvement of Leucine in tRNA Modification (Maurille Fournier and Alan Peterkofsky).

It has previously been shown that when "relaxed-control" E. coli is deprived of methionine, new species of tRNA (detected by column chromatography) are formed. Since methionine is the source of methyl-groups of tRNA, these new species are methyl-deficient. We have now found that new species of tRNA also accumulate when "relaxed control" E. coli are starved of leucine. New RNA synthesis is necessary for the phenomenon since the appearance of new species is prevented by leucine starvation of stringent control cells or uracil starvation of "relaxed control" cells. Leucine starvation leads to formation of new species that accept the amino acids leucine, arginine and histidine. This distribution suggests that leucine is involved in a modification reaction specific for those tRNA's that recognize codons beginning with C. This is in keeping with previous data from this and other laboratories showing that tRNA's recognizing codons beginning with U contain isopentenyl adenosine and those recognizing codons beginning with A contain N-(purin-6-ylcarbamoyl)-threonine. Further studies on these new species of tRNA will continue.

D. The Biochemistry of N-(purin-6-ylcarbamoyl)-threonine in tRNA (Donald Powers and Alan Peterkofsky).

The most recently discovered minor constituent in tRNA is N-(purin-6-ylcarbamoyl)-threonine (PCT). Since it has been found in the tRNA for the amino acids isoleucine, methionine, serine and lysine, it has been suggested that its occurrence will be restricted to those tRNA's whose codons begin with A. We have initiated a program to study the biosynthesis and distribution of PCT in tRNA. By exposure of a "relaxed control" threonine auxotroph of E. coli to threonine under conditions where RNA synthesis is allowed and protein synthesis is blocked, we have been able to specifically incorporate label from radioactive threonine into PCT. This is evidence that threonine is at least a partial precursor of PCT. Our plan for the immediate future is to utilize specifically labeled tRNA to deduce which species of tRNA contain PCT. This should allow us to test the distribution hypothesis mentioned above.

E. In vitro Transfer RNA Synthesis and Modification (Maurille Fournier, B. P. Doctor and Alan Peterkofsky)

We have recently initiated a program to purify those regions of E. coli DNA carrying the information for tRNA synthesis (tRNA cistrons). Our interest in this area is threefold: (a) We are interested in studying the factors controlling the synthesis of tRNA at the transcriptional level; (b) we would like to know the nature of precursor tRNA's if such exist; (c) we wish to study those in vitro modification reactions that, thus far, have not been amenable to examination. By a DNA-RNA hybridization procedure, we have been able to prepare enriched tRNA cistrons. These tRNA cistrons were used as a template with RNA polymerase. Further studies in this area are in progress.

Honors and Awards: None.

Publications:

Fournier, M., Doctor, B. P., and Peterkofsky, A.: Unique transfer RNA subspecies formed by leucine starvation of relaxed control E. coli. Fed. Proc. 29: 468, 1969.

Fournier, M., Brenner, D. J., Peterkofsky, A., and Doctor, B. P.: In vitro RNA synthesis using purified E. coli tRNA cistrons. British Biophysical Society, Jan. 1971.

Gonano, F., Stern, R., Littauer, U., Fleissner, E., and Peterkofsky, A.: tRNA meti-deficienti nella sintesi proteica. Italian Molecular Biology Society, Rome, 1970.

Litwack, M. and Peterkofsky, A.: Transfer RNA deficient in N^6-(Δ^2-isopentenyl) adenosine due to mevalonic acid limitation. Biochemistry 10: 994-1006, 1971.

Marmor, J. B., Dickerman, H. W. and Peterkofsky, A.: Studies in methyl-deficient methionine transfer ribonucleic acid from Escherichia coli. J. Biol. Chem. 246: 3464-3473, 1971.

Serial No. NHLI-127
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Genes for Neuronal Properties Expressed in Neuroblastoma x L Cell Hybrids.

Previous Serial Number: None.

Principal Investigator: John D. Minna.

Other Investigators: Marshall Nirenberg, Takehiko Amano, and Devera Glazer

Cooperating Units: Phillip G. Nelson and John H. Peacock, Behavioral Biology Branch, National Institute of Child Health and Human Development.

Project Description:

Mutant clones of mouse neuroblastoma were selected by mutagenesis and exposure to 6-thioguanine. The electrically excitable neuroblastoma cells were fused with electrically passive L cells having a hitherto undescribed electrical marker. Hybrid clones, examined 10-40 generations after fusion, were found to be electrically excitable. The results show that at least part of the genetic information from neuron differentiation can be functionally expressed in neuroblastoma x L cell hybrids. No evidence for a pleiotropic repressor terminating neuron differentiation was found. In fact, most N x L cell hybrids were more active electrically than the parental neuroblastoma line. Somatic cell hybridization applied to normal neuroblasts should provide a relatively simple means of establishing clonal lines of cells derived from different types of neurons. The levels of acetylcholinesterase of N x L cell hybrid clones were determined 30-50 cell generations after cells were fused. The neuroblastoma parent and all N x L cell hybrids contained acetylcholinesterase; appreciable amounts of enzyme were not detected in the L cell parent or in L x L hybrids. The results show that neuroblastoma genes for acetylcholinesterase continue to be expressed after fusion.

Results obtained with neuroblastoma clearly show that rapidly dividing cells still retain the ability to express neuronal functions and that some neuronal genes remain active in somatic cell hybrids. The possibility that neurons may be capable of initiating a program of neuron differentiation in recipient cells also deserves consideration.

Honors and Awards: None.

Publications:

Minna, John, Nelson, Phillip, Peacock, John, Glazer, Devera and Nirenberg, Marshall: Genes for neuronal properties expressed in neuroblastoma x L cell hybrids. Proc. Natl. Acad. Sci. 68: 234-239, 1971.

Nelson, P. G., Peacock, H. H., Amano, T. and Minna, J.: Electrogenesis in mouse neuroblastoma cells in vitro. J. Cell. Physiol., in press.

Nelson, P. G., Peacock, H. H., and Amano, T.: Responses of neuroblastoma cells to iontophoretically applied acetylcholine. J. Cell. Physiol., in press.

Serial No. NHLI-128
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Acetylcholinesterase in Neuroblastoma Cells.

Previous Serial Number: None

Principal Investigator: Arthur J. Blume

Other Investigator: Marshall Nirenberg

Cooperating Units: None

Project Description:

We have previously shown that neuroblastoma cells contain acetylcholinesterase and both excitatory and inhibitory acetylcholine receptors and that these factors respond to regulatory mechanisms in vitro. Thus, purification and characterization of the factors were initiated to define the relationship between acetylcholinesterase and acetylcholine receptors and the nature of the regulatory mechanisms. Acetylcholinesterase was found to be associated with membranes; however, the enzyme now has been purified extensively and is now being characterized.

Honors and Awards: None.

Publications:

Blume, A., Gilbert, F., Wilson, S., Farber, J., Rosenberg, R. and Nirenberg, M.: Regulation of acetylcholinesterase in neuroblastoma cells. Proc. Natl. Acad. Sci. U.S.A. 67: 786-792, 1970.

Serial No. NHLI-129
1. Biochemical Genetics
2. Molecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cyclic AMP Metabolism in Neuronal and Glial Clonal Cell Lines

Previous Serial Number: None

Principal Investigator: Alfred G. Gilman

Other Investigator: Marshall Nirenberg

Cooperating Units: None

Project Description:

A simple, rapid assay for adenosine 3'5'-cyclic monophosphate was developed that is based upon competition between labeled and unlabeled cAMP for a protein, presumably a cAMP-dependent protein kinase. The nucleotide-protein complex is then adsorbed and washed on a cellulose ester filter. The assay is sensitive to 0.05-0.10 pmoles of cAMP, and is being used to explore cAMP metabolism in neuroblastoma and other cell types that are grown in vitro.

Honors and Awards: None

Publications:

Gilman, A.G.: A protein binding assay for adenosine 3'5'-monophosphate. Proc. Natl. Acad. Sci. 67: 305-312, 1970.

Rall, T. W. and Gilman, A.G.: The role of cyclic AMP in the nervous system. Neurosciences Research Program Bulletin 8: 221-323, 1970.


Endocrinology
Chemistry
Chemical
Biochemistry

ANNUAL REPORT OF THE
LABORATORY OF BIOCHEMISTRY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

SECTION ON ENZYMES

Research in the Section on Enzymes has been concerned with the cellular regulation of nitrogen metabolism, amino acid dissimilation, the mechanism of action of vitamine B_{12} coenzymes, the anaerobic dissimilation of nicotinic acid and the regulation of homocysteine biosynthesis.

Regulation of Glutamine Metabolism. (a) Glutamine synthetase. Previous studies demonstrated that the activity of glutamine synthetase (GS) in E. coli is regulated by adenylylation and deadenylylation of a single tyrosyl hydroxyl group on each one of the enzyme's 12 subunits (Reactions 1 and 2).

$$GS + 12\ ATP \rightleftharpoons GS\ (AMP)_{12} + 12\ PPi \quad (1)$$

$$GS\ (AMP)_{12} + 12\ Pi \rightleftharpoons 12\ ADP \quad (2)$$

Adenylylation is accompanied by changes in catalytic potential, in divalent cation requirement (from Mg^{++} to Mn^{++}), in pH optimum, and in sensitivity to inhibition by end products of glutamine metabolism. The average state of adenylylation is determined by the relative rates of reactions 1 and 2 which are modulated in reciprocal fashion by concentrations of glutamine, α-ketoglutarate, UTP and ATP. It has now been established that deadenylylation involves phosphorolysis (rather than hydrolysis as previously assumed) of the phosphodiester linkage binding AMP to enzyme, yielding ADP as the reaction product (reaction 2). The energy of the adenylyltyrosine bond is thus conserved in the generation of a pyrophosphate bond. It was further established that a single protein complex (P_I) containing one adenylyltransferase subunit (ATase subunit, MW = 70,000 MW) and a subunit required for deadenylylation (DA subunit, MW = 60,000) is involved in catalyzing both reactions 1 and 2. Either P_I alone or its ATase subunit by themselves can catalyze reaction 1; however, intact P_I is required for reaction 2, and its catalytic potential and sensitivity to metabolite control is dependent upon a second protein component (P_{II}) of 50,000 MW. With different enzyme preparations (P_I plus P_{II}), the sensitivity of reaction 2 to activation by α-ketoglutarate, ATP and UTP and its inhibitability by glutamine are variable. The possibility that the activity of P_{II} is also modulated by covalent attachment of a nucleotide is indicated by its inactivation upon exposure to snake venom phosphodiesterase.

It has been established that E. coli contains multiple molecular forms (hybrid molecules) of glutamine synthetase that differ from one another in the number (0 to 12) and distribution of adenylylated subunits. Heterotropic interaction between adenylylated and unadenylylated subunits in these hybrid enzyme molecules lead to marked changes in catalytic potential, in affinities for substrates and in susceptibility to urea denaturation. Hybrids produced by subunit dissociation and reassociation

of mixtures of adenylylated and unadenylylated enzymes are indistinguishable from naturally occurring hybrid forms produced by partial adenylylation or deadenylylative reactions.

From the relationship between specific activity of Co^{++} activated enzyme and the average state of adenylylation it is deduced that adenylylation of a subunit leads to its inactivation and (because of heterotropic interactions) to nearly complete inactivation of unadenylylated subunits that are in direct contact with the adenylylated subunit. It is a consequence of such heterologous interactions that the greatest effect on catalytic activity is achieved by the adenylylation of only 1 to 3 of the enzyme's 12 subunits and almost complete inactivation is obtained when only 9 subunits are adenylylated. This non-linear relationship between catalytic potential and state of adenylylation could be of physiological significance since the affinity of the adenylyltransferase for glutamine synthetase decreases rapidly with state of adenylylation, making complete adenylylation difficult.

Differential effects of various divalent cations (i.e., Co^{++}, Mg^{++}, Mn^{++}, Ca^{++} and Zn^{++}) on pH optimum, affinity for substrates, catalytic potential, inhibitability by end products of glutamine metabolism and U.V. spectrum of glutamine synthetase indicate that each cation provokes a unique conformation of the enzyme.

Calorimetric studies of the interaction between divalent cations and enzyme (carried out in collaboration with Dr. P.D. Ross of NIAMD) show that 2 protons are displaced from the enzyme during the binding of each of the first 12 equivalents of either Mn^{++} or Mg^{++} to the enzyme. One proton is released instantaneously and the second in a slow process (half time = 0.5 - 0.9 min) probably involving a conformational change in the protein. The kinetics of this slow reaction are similar to those of the spectral shifts that accompany binding of Mn^{++} or Mg^{++}. Despite these rather large perturbations in the microenvironment of certain aromatic amino acids that accompany binding of divalent cations to glutamine synthetase, these cations have no demonstrable effect on the secondary or quaternary structure as determined by circular dichroism (CD) and optical rotatory dispersion measurements (310-218 nm). From CD measurements, it was estimated that the α-helical content of native glutamine synthetase is 37%.

In an effort to distinguish between unique configurations of unadenylylated enzyme ($E_{\bar{12}}$), adenylylated enzyme ($E_{\bar{12}}$) and their dissociated subunits, antibodies were prepared to each species of enzyme and subunits. $E_{\bar{12}}$ and $E_{\bar{12}}$ could not be distinguished from one another on the basis of complement fixation using antiserum to either form of enzyme. The results reinforce conclusions based on other data that adenylylation does not provoke a substantial change in enzyme structure. However, differences in behavior of antibodies prepared to subunits indicate that adenylylation introduces a distinct antigenic determinant on the enzyme. A cross reaction between antibodies of E. coli glutamine synthetase and the synthetases from Salmonella typhimurium, Klebsiella pneumoniae, Pseudomonas putida and Azotobacter vinelandii indicate a significant degree of homology bewteen these enzymes. No cross reaction was obtained with glutamine synthetases

from various species of Bacilli, Clostridia, Streptomyces, Neurospora, Methanosarcina or Acanthomoeba. (b) Glutamate Synthetase. Tempest, et al. (Biochem. J. 117, 405 (1970)) recently reported the discovery of a new enzyme, glutamate synthetase, that catalyzes the TPNH dependent conversion of α-ketoglutarate and glutamine to two moles of glutamate. (reaction 1).

$$\text{Glutamine} + \alpha\text{-ketoglutarate} + \text{TPNH} \rightarrow 2 \text{ glutamate} + \text{TPN} \quad (1)$$

$$\text{Glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + \text{Pi} \quad (2)$$

$$\alpha\text{-Ketoglutarate} + NH_4^+ + \text{ATP} + \text{TPNH} \rightarrow \text{glutamate} + \text{ADP+TPN+Pi} \quad (3)$$

When coupled with glutamine synthesis (reaction 2) this new reaction leads to the overall reaction 3 which represents a new highly exergonic pathway for the synthesis of glutamate. The new glutamate synthetase has now been purified to near homogeneity from extracts of E. coli: It has a molecular weight of about 750,000 as determined by ultracentrifugation and gel filtration. The S_{20w} = 22S; pH optimum = 7.8. The protein contained 1 mole of bound flavin (FAD + FMN) per 200 gms which was reduced by TPNH but not by DPNH. The apparent dissociation constants (Kms) for glutamine and α-keto-glutarate are 170 and 30 μM respectively. These Kms and the low Kms for the substrates of glutamine synthetase permit rapid synthesis of glutamate by reaction 3 at concentrations of NH_4^+ that preclude significant glutamate formation by the classical glutamate dehydrogenase. Therefore the new pathway of glutamate synthesis becomes important when the intracellular concentration of NH_4^+ is low. Its role in glutamate synthesis is further indicated by the fact that its formation is repressed by high concentration of glutamate. It is noteworthy that in this new pathway glutamine is a precursor of glutamate synthesis, and therefore of all amino acids derived from glutamate either directly or via transamination reactions. The previously unexplained inhibitions of glutamine synthetase by alanine, glycine or serine are now recognized as simple cases of feedback inhibition of the first enzyme in the biosynthetic pathway by ultimate end products.
(c) Glutaminase. It is known that E. coli contains two glutaminases with different pH optima (pH 5 and pH 7). As part of the study on regulation of nitrogen metabolism in E. coli, the properties of these glutaminases were investigated. Growth studies with E. coli B showed that the pH 5 enzyme increases markedly during stationary phase whereas the pH 7 enzyme is highest during the log phase and early stationary phase. Preliminary experiments indicate that highest activity of the pH 7 enzyme is obtained in the presence of excess nitrogen and growth is limited by carbohydrate supply. The pH 7 enzyme has been purified over 1700 fold from crude extracts. It is a cold-labile enzyme and is inactivated at 0° and is partially reactivated by either glutamine or glutamate. The enzyme is stabilized by glutamate and borate. It exhibits complex kinetics suggestive of cooperative substrate interactions and has almost no activity at pH 5.

Amino Acid Metabolism. The B_{12} coenzyme dependent lysine mutases of Clostridium sticklandii and Clostridium M-F have been studied in detail as regards reaction mechanism and characterization of the proteins. Both α-D-lysine mutase and L-β-lysine mutase consist of two dissimilar protein moieties, and B_{12} coenzyme serves as carrier of the migrating hydrogen in each reaction. The cobalamide protein moiety of α-D-lysine mutase contains

pyridoxal phosphate and this binds the ω-amino group of the amino acid substrates and probably serves as intermediate amino group carrier in the mutase reaction. L-α-lysine mutase appears to contain pyruvate as its carbonyl compound rather than pyridoxal phosphate. Both of the mutases are much more complicated in their requirements, such as activation by ATP, than are other B_{12} coenzyme linked enzymes previously studied. Their complex properties are probably involved in regulation of the two parallel metabolic pathways-fermentation of D-α-lysine and L-α-lysine in which they participate.

The product of L-β-lysine mutase, 3,5-diaminohexanoic acid, contains two asymmetric centers (C-3 and C-5) giving rise to two pairs of enantiomers that differ in their melting points. Since a knowledge of the steriochemistry of the enzymic product would aid in understanding the mechanism of the mutase reaction, these isomers were obtained by chemical synthesis. It has been established that only the low melting isomer is enzymatically active. From NMR spectral studies of benzoylated amino lactam derivatives of the isomeric amino acid it was deduced that the enzymatically active isomer has the erythro configuration. Since the β-lysine substrate has the S-configuration the new asymmetric center, C-5, in 3,5-diaminohexanoic acid must therefore have an S-configuration.

The peptide growth factor requirement of Clostridium sticklandii is satisfied by a fraction isolated from a casein digest that contains only two major acidic peptide components. The amino acid composition of this peptide fraction is glutamic acid, valine, leucine, isoleucine, alanine and some serine and threonine. Based on molecular weight estimations, the active peptide(s) should consist of 5 to 7 amino acid residues. Experiments are currently in progress to achieve final isolation and determination of structure of the required peptide.

The ATP-forming glycine reductase system of C. sticklandii, after fractionation into three soluble highly purified protein components still shows an absolute requirement for orthophosphate in order to reduce glycine to acetate and ammonia with dithiothreitol as electron donor. Marked sensitivity to hydroxylamine suggests a carbonyl compound cofactor is involved.

Methane biosynthesis. Since the early reduction steps in methane formation from carbon dioxide and formate are completely unknown, ^{14}C formate reduction to methane by Methanococcus vannielii extracts is being investigated. A nucleotide fraction in the extracts, which becomes highly radioactive during the reaction, has been purified and shown to serve as a precursor of ^{14}C labeled methane. Chemical characterization of this radioactive material, now in progress, should determine whether it has an intermediary role in the process. Marked stimulation of methane biosynthesis from formate by several one-carbon derivatives of uracil suggests that the presumed intermediate may be a modified uridine nucleotide.

Anaerobic dissimilation of nicotinic acid. In the nicotinic acid fermentation pathway the obligatory roles of two inducible enzymes which together rearrange the carbon skeleton of the original substrate to a symmetrical intermediate account for the results of earlier isotope studies. One of these enzymes, the B_{12} coenzyme-dependent α-methyleneglutarate mutase, catalyzes

a carbon-carbon bond cleavage reaction and this, followed by a second isomerization catalyzed by methylitaconate isomerase, forms the symmetrical dicarboxylic acid, dimethylmaleic acid. The mechanisms of the hydrogen transfers catalyzed by both enzymes have been studied.

Regulation of methionine biosynthesis. Previous work in this laboratory has shown that the synthesis of homocysteine, the immediate precursor of methionine, is accomplished in 3 sequential steps, which differ slightly in different microorganisms, and are catalyzed by: a) homoserine transacylase, utilizing succinyl CoA in Salmonella and acetyl CoA in Neurospora; b) cystathionine γ-synthase; and c) β-cystathionase. In Neurospora the cystathionine synthase, which had been shown to be subject to end product inhibition by S-adenosylmethionine, has now been found to be completely inactive in the absence of polyglutamate derivatives of N^5-methyltetrahydrofolate. This allosteric activation serves to prevent over-production of homocysteine in the absence of the methylfolate needed for its conversion to methionine. It is noteworthy that in Neurospora it is the second enzyme of the homocysteine synthetic pathway that is subject to regulation. The intermediary role of cystathionine in methionine synthesis in yeast is still uncertain. Now significant cystathionine synthase activity can be detected in extracts of yeast protoplasts, but the reaction is not affected by addition of methylfolates and is slower than the direct formation of homocysteine from acetylhomoserine and sulfide. In Salmonella also, where the first enzyme of the pathway is subject to end product inhibition, a regulatory role for methylfolates could not be demonstrated. The mechanism regulating the synthesis of the transsuccinylase in Salmonella appears to be different from that regulating the second and third enzymes. When the growth rate of a mutant which responds to either methionine or vitamin B_{12} is slowed in a chemostat by limiting the concentration of methionine the second and third enzymes are derepressed in a normal fashion, but it is virtually impossible to derepress the trans-succinylase. This result is not observed with vitamin B_{12} limitation, suggesting that exogenous methionine is a preferential source of a component involved in controlling the synthesis of the transsuccinylase.

Annual Report of the
Section on Cellular Physiology
Laboratory of Biochemistry
National Heart and Lung Institute
July 1, 1970 through June 30, 1971

The research program of the Section on Cellular Physiology continues to be directed toward two broad areas of biochemistry and cellular physiology; structure and structure-function relationships of proteins on the one hand and the role of cell membranes in synthetic activities of cells on the other, encompassing programs in the areas of (1) the structure and biochemical activities of the proteins of the contractile system of muscle; (2) the structure of fibrinogen; (3) radiation damage in proteins; and (4) the mechanism of protein synthesis and its relationship to cell structure. The following is a resume of some major developments in the research of the section.

Proteins of the contractile system of muscle.

Myosin-actin interactions:

It is now generally recognized that contraction of muscle involves the interaction of the two proteins, actin and myosin, with ATP. Clearly, it is of importance to determine the strength of binding and the binding ratio of actin to myosin both in the presence and absence of ATP. While there is a voluminous literature on the characteristics of actin-myosin complexes, no stoichiometry can be deduced for this system under conditions of physiological ionic strength where both proteins exist as aggregates. However, heavy meromyosin (HMM) a product of brief tryptic digestion of myosin constituting about 75% of the mass of the myosin molecule is soluble and monomeric at low ionic strength and its binding to actin can be studied in the analytical ultracentrifuge.

Actin under conditions of physiological ionic strength, or higher, exists as a double stranded linear polymer and rapidly sediments in the ultracentrifuge. In binding experiments with HMM any unbound HMM is left in the supernatant and can be quantitated with the photoelectric scanning system. Such studies under a variety of conditions of ionic strength, temperature and initial actin concentrations, have demonstrated that the maximum molar binding ratio of HMM:actin monomer in the absence to ATP is 1:2. Furthermore, under all these conditions, the binding constant was too high to measure -- greater than $2X10^6M$.

This binding is many fold stronger than that in the presence of ATP as deduced from kinetic measurements. The binding ratio of 1:2 is in agreement with the fact that myosin and HMM are 2-headed molecules so that apparently each head binds to a separate actin monomer.

In the presence of ATP actin strongly activates the HMM ATPase and therefore the extent of actin-HMM binding can be estimated from kinetic measurements. These measurements suggest that in the presence of ATP the binding between actin and HMM is much weaker than in the absence of ATP. In an effort to directly examine the binding in the presence of ATP, the analytical

ultracentrifuge was used. Unexpectedly it was found that under conditions where kinetic measurements suggest that the actin and HMM are completely complexed, the ultracentrifuge showed that 50% of the HMM was in fact dissociated from the actin. This suggests that during the cycle of actin-HMM interaction, much of the HMM exists in a "refractory" form in which it cannot bind to actin. Since HMM is a 2-headed molecule, it is quite likely that only one head of the molecule emerges from its refractory period at a time, so that, in effect, only one head of the HMM binds to actin in the presence of ATP. This would of course contrast with the situation in the absence of ATP where both heads bind simultaneously and this difference in the number of heads binding might account for the strong binding which occurs in the absence of ATP as opposed to the weak binding in the presence of ATP.

The observation that much of the HMM is in a refractory form where it cannot bind to actin in the presence of ATP may have significant implications for the situation in vivo. The flexability of contracting muscle as opposed to rigor muscle may occur because in the rigor state all of the myosin bridges are bound to the actin filament whereas in contracting muscle only a fraction of these bridges are bound. In support of this hypothesis X-ray diffraction studies of contracting muscle show that less than 50% of the myosin bridges are closely associated with actin. Therefore the occurrence of a refractory period during which the myosin molecule cannot bind to actin may play a key role in the cyclic interaction of actin and myosin which causes contraction in vivo.

Actin:
G-Actin, the major protein of the thin filaments in the contractile apparatus of muscle -- and, apparently, of other cells as well -- has a molecular weight of 45,000 and consists of a sequence of 410 amino acid residues. As an initial goal in elucidating the primary structure of actin, it was decided to attempt isolation of all of the peptide fragments of actin resulting from cyanogen bromide cleavage of the molecule. Cyanogen bromide specifically attacks methionyl residues cleaving the adjacent peptide bond and converting the methionine to carboxyl terminal homoserine of the resulting peptide fragments. Subsequent isolation of all of the methionine containing peptides resulting from hydrolysis of actin by trypsin should result in a set of overlapping peptides. Matching up of these two sets of peptides should then result in the ordering of the cyanogen bromide fragments and provide a base for further sequence analysis. There are sixteen methionine residues in actin leading to seventeen cyanogen bromide fragments. The previous report dealt with the isolation of all seventeen of these and the results have been published. With respect to the sixteen overlaps all but one have been successfully identified. Although the missing overlap can be inferred, efforts continue to positively identify it. With the placing of the seventeen cyanogen bromide peptides in sequence the initial goal of this project is complete. From this information and sequence studies in another laboratory we now have about 70% of the 410 amino acid residues of actin in sequence.

Myosin:
The myosin molecule is composed to two identical polypeptide chains each of 200,000 molecular weight. In addition about 15% of the mass of all

native myosin preparations consist of smaller polypeptide chains of about 20,000 molecular weight. It has not been possible, so far, to separate the latter material from the large chains except under denaturing conditions. While the small chains are widely regarded as a functional part of the myosin molecule, this has not been proved. No stoichiometry in the structure has been demonstrated, a problem that is complicated by the fact that this low molecular weight fraction is composed of four components in unequal amounts. Conflicting figures in the literature are probably confused by the fact that myosin prepared by "standard" laboratory procedures always contains 5-10% of low molecular weight contaminants readily removable by any one of three chromatographic procedures. Some time ago, before the presence of the small chains in myosin was recognized it was observed in this laboratory that myosin possessed histidine as an amino terminal group. Subsequently, another laboratory reported the presence of a blocked N-terminal, identified as acetyl serine. It has now been determined that the large chains of myosin possess the amino terminal histidine, while the small chain fraction possesses one component, readily separable from the rest by chromatography, which contains a blocked amino terminal, tentatively identified as acetyl serine. In addition, the small chain fraction possesses amino terminal alanine, about six-tenths of a mole per mole of small chains, and a minor amount, about 0.1 mole, of either aspartic or glutamic acid. Disc gel electrophoresis of the small chains shows the presence of two major and two minor components. With the high recovery of N-terminal alanine it must be assumed that two of the four components have amino terminal alanine and the other two recognized N-terminal structures account for the other two components.

Myosin from human blood platelets:

Starting with human platelet concentrates, two myosin-like proteins have been isolated. Both proteins, like myosin, have ATPase activity that is stimulated by Ca^{++} and EDTA and inhibited by Mg^{++}. Both proteins bind to rabbit skeletal actin and are released by Mg-ATP. In the details of their enzyme activities these two proteins resemble smooth muscle myosin. The two proteins differ in molecular weight. On disc gel electrophoresis in the presence of sodium dodecylsulfate the heavier of the two proteins, coelectrophoreses with the heavy chains (200,000) of rabbit skeletal myosin. The lighter of the two proteins shows a subunit molecular weight of about 100,000 in the same electrophoretic system.

Electron microscopic confirmation of structural similarities to myosin has also been obtained. The heavier of the platelet proteins forms aggregates at low salt concentration which are similar to the "thick filaments" formed by muscle myosin. Moreover, the lighter species forms the "arrowhead" structures with rabbit skeletal actin characteristic of the interaction of skeletal muscle heavy meromyosin and actin.

Annual Report of the
Section on Cellular Biochemistry and Ultrastructure
Laboratory of Biochemistry
National Heart and Lung Institute
July 1, 1970 through June 30, 1971

Research has continued in the three related areas of: 1) the molecular and ultrastructural basis of movement in motile, non-muscle cells, 2) the biochemistry and ultrastructure of the plasma membrane, and 3) phagocytosis and pinocytosis. In general the soil amoeba, Acanthamoeba castellanii, has provided the experimental material but none of the phenomena under study is unique to amoebae. The amoeba does provide a readily cultured, homogeneous population of cells which are relatively highly motile, which are nutritionally exclusively dependent on phagocytosis and pinocytosis, and which may have a simpler plasma membrane than mammalian cells.

(1) The analogy between amoeba actin (obtained as a pure protein last year in this laboratory) and muscle actin has been developed further by demonstrating major similarities in the amino acid composition of three of the peptides produced by cleavage of each protein by cyanogen bromide. One of these, the peptide that contains the unusual amino acid 3-methylhistidine, may have identical amino acid composition in the amoeba and rabbit actins.

We have now purified some 200-fold an amoeba ATPase with enzymatic properties essentially identical to those of muscle myosin; i.e., a K^+, EDTA-stimulated ATPase that is also active as a Ca^{++}-ATPase but which is inhibited by Na^+ and Mg^{++}. The amoeba ATPase is activated 30-fold by the addition of muscle actin in the presence of Mg^{++}. Although myosin-like ATPases have previously been found in other motile cells this is the first instance in which it has been possible to show activation of a non-muscle ATPase by actin. A very interesting observation has been that the amoeba ATPase seems to have a molecular weight of about 130,000 which is much lower than the molecular weights of all other myosin-like ATPases (about 450,000). This difference may explain the inability thus far to demonstrate the presence of thick, myosin-like filaments in the amoebae. Thin, actin filaments are present.

The biochemical and ultrastructural studies with the Acanthamoeba have provided some of the strongest evidence for the fundamental similarities between the processes of cell motility and muscle contraction.

(2) Last year we reported the procedure for the isolation of highly purified amoeba plasma membranes which contain a very active alkaline phosphatase, and described the ratio of lipid to protein and the composition of the phospholipids, sterols and glycerides. We have now found that the amoeba plasma membrane contains a uniquely high concentration of non-lipid phosphorus (1 μmole/mg protein) and a very high concentration of carbohydrate (at least 1 μmole of "glucose" equivalents/mg protein). The delipidated membrane has been fractionated into a high molecular weight fraction which contains all of the carbohydrate and non-lipid P and a small percentage of the protein, and a low molecular weight fraction which contains only protein. The protein fraction seems to contain relatively few components (only two or three bands

are seen in polyacrylamide gel electrophoresis of the whole membranes) none of which has a molecular weight greater than 15,000 and half of which have molecular weights less than 5000 by the usual criteria. The carbohydrate contains glucose, mannose, xylose, and several unidentified sugars. The polymer that contains the non-lipid P has not been identified but the P is not released upon hydrolysis in 3N HCl at 100° for 3 hours.

As originally speculated from functional considerations, it now seems probable that the amoeba plasma membrane is indeed much simpler in protein composition than the corresponding membranes of mammalian and bacterial cells and thus may provide a very useful system for establishing the molecular organization of a biological membrane.

(3) Both the plasma membrane and the motile process are involved in endocytosis. Recent biochemical and ultrastructural studies have confirmed our previous impression that solute molecules enter the amoeba only by pinocytosis. The magnitude of this continuous process is such that we estimate from biochemical and electron microscopic data that the amoeba surface membrane turns over approximately once a minute. Scanning electron microscopy has yielded striking three dimensional images of the amoebae, illuminated the mechanisms by which they capture particles during phagocytosis, and shown the relationship of the amoeba to the substrate during motion.

Serial No. NHLI-130
1. Laboratory of Biochemistry
2. Section on Enzymes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Bacterial Purine and Pyrimidine Base and Nucleoside Utilization

Previous Serial Number: NHLI-200

Principal Investigator: Joy Hochstadt-Ozer

Other Investigators: None

Cooperating Units: M. Cashel, NINDS
M. Levinthal, NIAMD

Project Description:

Objectives: Previous studies from this laboratory indicated that phosphoribosyltransferases (PRT'ases) in Bacillus subtilis might be involved in purine uptake. Isolation, characterization, and study of regulation of adenine PRT'ase in E. coli showed that this enzyme is membrane-associated, can be released from the pericytoplasmic space upon osmotic shock of viable cells, is responsible for adenine transport by a group translocation mechanism and that both PRT'ase activity and uptake are regulated by 5' nucleotides. The present project is concerned with extending the studies so far concentrating on adenine toward the elucidation of the mechanism, regulation and coordination of all nucleic acid precursors, and to examine the genetic and membrane topographic and/or functional interrelationship of the carrier and/or enzymes responsible for the control, uptake and utilization of nucleotide precursors. This system seems furthermore to be ideal as a model for study of nascent membrane functional units as appropriate genetic reagents and reconstitution techniques are being developed in it.

Major Findings:

Adenine Phosphoribosyltransferase: Regulation by combinations of nucleotides: A variety of combinations of two '5 nucleotides were tested to determine possible cooperativity between effectors. 6-OH nucleotides could antagonize the effect 6-NH_2 nucleotides to a small but significant extent. The mechanism of antagonism appeared to be by competing at the same inhibitory site (possibly the catalytic site since '5 nucleotides each inhibit by competing with the substrate PRPP).

Guanine Hypoxanthine-Xanthine Phosphoribosyltransferase(s): Further purification of a several hundred fold-purified enzyme preparation on Ecteola-Cellulose, has led to the identification of at least three protein

peaks each exhibiting all three phosphoribosyl activities. Since these activities were not separable by gel filtration chromatography they appear to have similar molecular weights. Genetic analysis to determine whether these represent modified forms of a single gene product or separate gene products are in progress. Each peak of activity is being analysed for possible differences in enzymatic and regulatory behavior. Major differences have not been observed with respect to hypoxanthine as substrate. Studies to determine differences with respect to guanine, xanthine, 8-azaguanine, and 6 Mercaptopurine as substrate are in progress.

Mutants of Purine Utilization. Though analog resistant mutants of purine utilization have been reported for E. coli these have been found to be unstable and to revert to PRT'ase activity unless maintained on the selective medium. The occurrence of the several (possibly interconvertable) PRT'ase forms may contribute to this genetic "instability" in E. coli. Neither drug selection alone nor purine plus penicillin selection produced stable mutants from either normal or mutagenized stocks. Combination of drug resistance, purine-penicillin selection and screening by replica plating of mutagenized cells in sequence is currently being employed as a means of selecting double or triple mutants should such multiple mutations be requisite to loss of a purine PRT'ase activity. Alternatively use of phage transduction for the construction of an appropriate deletion if the PRT'ase determinants are clustered has been undertaken in collaboration with Dr. Mark Levinthal, Laboratory of Molecular Biology, NIAMD. For this, a Salmonella typhimurium strain carrying a deletion in the Proline A B region in which 8-Azaguanine resistance was fortuitously found, was chosen as starting material. Under appropriate conditions hypoxanthine uptake levels and PRT'ase levels in this strain are consistent with the PRT'ase functioning in utilization in a manner similar to that found for E. coli. Characterization of Salmonella enzyme and its interspecific transfer to E. coli may provide a means of genetic analysis without loss of PRT'ase activity. The possibility that purine PRT'ases represent an essential function in E. coli is thus being considered, and in addition to utilization of Salmonella reagents, conditional lethal mutants are being sought in E. coli.

Mechanism of Uptake of Adenosine and Inosine. The mechanism of uptake of adenosine (and inosine) was studied in isolated membranes and found to have two steps: 1) Cleavage to adenine (and hypoxanthine) and ribose-1-P by nucleoside phosphorylases and 2) Group translocation by adenine (hypoxanthine) phosphoribosyltransferases. Though the kinetic and regulatory parameters of the adenine and hypoxanthine systems differ markedly from one another, the response to any effector or rate limiting condition for adenine (hypoxanthine) uptake was identical to its effect on adenosine (inosine) uptake. 5' Nuclectides are inhibitors in both systems.

Mechanism of Cytosine, Cytidine, Uracil, and Uridine Uptake. Uracil PRT'ase seems to be involved with uracil uptake by a mechanism similar to adenine uptake. Cytosine PRT'ase is lacking in E. coli and cytosine is probably taken up as uracil. Cytidine and uridine uptake do not follow cytosine and uracil uptake as identically as adenosine and inosine uptake

follow adenine and hypoxanthine uptake, respectively. Nucleoside phosphorylase for cytidine was found to be lacking in our strain of E. coli. '5 Nucleotides are inhibitors of all nucleoside and base uptake by purified membranes, however.

Effect of ppGpp on Uptake and Enzymes of Nucleic Acid Precursors Metabolism and Interconversion. The production of ppGpp ("Magic Spot") by stringent strains of E. coli and its effect, inhibition of RNA synthesis, has been well established by Dr. Cashel in the Laboratory of Molecular Biology, NINDS. Dr. Cashel, in collaboration with the laboratory of Dr. Ira Pastan, has recently found ppGpp exerts a regulatory effect on protein synthesis in vitro. Possible coordination of regulation of gene action and of existing enzymatic activities by this compound in effecting complete control of nucleic acid synthesis has been studied by determination of its effect on uptake of bases and nucleosides and enzymes of nucleotide synthesis and interconversion. ppGpp is a potent inhibitor of adenine, adenosine, hypoxanthine, inosine, guanosine, and uridine uptake but not uracil, cytosine, and cytidine uptake. It is a potent inhibitor of adenine, guanine, and hypoxanthine PRT'ase activities but not purine nucleoside phosphorylases. It is an inhibitor of adenosine deaminase and uridine phosphorylase but not uracil PRT'ase. The level of inhibition on guanine utilization or prevention of deamination of intracellular adenine derivatives (otherwise convertable to guanine nucleotides) when compared with intracellular ppGpp levels in stringent cells deprived of an essential amino acid can adequately account for immediate cessation of nucleic acid synthesis in such cells.

Dual Isotope Experiments. Commercial availability of uniformly labeled ^{14}C bases and nucleosides with specific activities greater than 10 times that commercially available at the beginning of this project (1968) have permitted the following experiments to be performed. Membrane vesicles were preloaded with either ^{3}H or ^{14}C adenine, adenosine, or hypoxanthine. Then membranes were washed free of extravesicular tracer and incubated with ^{14}C or ^{3}H adenine, adenosine, or hypoxanthine and PRPP. The results indicated that extravesicular labeled material is preferred to the preloaded material as a substrate for phosphorylation ('5 nucleotide synthesis).

Significance to Bio-Medical Research: This project investigates the nature and regulation of structure-function-relationships of cell membranes as involved in purine and pyrimidine utilization. Understanding of this organelle possessed by all living cells (and known to be altered in a number of pathologic states), is essential for a conceptual framework of cell function, both normal and pathologic. The experimental program utilizes enzyme-transport systems capable of assay both in situ (transport activity) and as homogeneous proteins in aqueous solutions (catalytic activity). Such independent assay, in the complex milieu of the membrane and in isolation has the advantage of allowing comparison of the properties of membrane components in the biological as well as in well defined environments while experimentally preserving the ability to monitor retention of at least one biologic function in either environment. The enzyme-transport systems themselves are those involved in utilization of nucleic acid

precursors and deficiency of one of them (hypoxanthine system) has been identified in man (Lesch-Nyhan Syndrome). The multiple clinical anomalies associated with absence of the hypoxanthine phosphoribosyltransferase (previously thought to act in a "salvage" capacity only) indicates the necessity of a further research into the role recycling of nucleic acid precursors play in the cell economy.

Proposed Course of Project:

A. Enzymes, Membranes, and Transport. Characterization of the multiple peaks of PRT'ase activity for each purine will be continued to determine the genetic or chemical determinant(s) conferring such differentiability.

B. Role of the PRT'ases in purine transport. Conditions will be determined to control the amount of PRT'ase associated with membrane vesicles during membrane isolation. The amount of enzyme and its state of association with the membrane will be correlated with the PRT'ase-membrane interaction necessary to carry out transport.

C. Reconstitution experiments. The ability of isolated enzyme to stimulate transport when added to isolated membrane vesicles and intact cells will be evaluated. Attention will be paid to the conditions necessary for such reconstitution. These experiments, where initial attempts to remove considerable PRT'ase from membranes, will precede reconstitution, should complement experiments mentioned above in B. Purified enzyme will also be reacted with membranes prepared from mutants lacking PRT'ase and uptake activity in an attempt to confer these functions on such mutant membrane preparations.

D. Comparative enzymology of PRT'ase derived from sonically disrupted cells and isolated from membrane vesicles. Using a number of specific probes usefu1 in determining protein structure and reactive groups (e.g., -SH reagents, tryosine reagents, lysine reagents, acetylation), the nature of PRT'ase-membrane association will be investigated. The protection to various reagents, conferred by membrane-enzyme association or conversely heightened reactivity for (a) PRT'ase activity, and (b) transport, will be investigated. The effect of nucleotide inhibitors on these interactions will be subsequently studied. Serological techniques will also be employed in evaluation of the nature of the membrane-PRT'ase interaction. Antibody to purified soluble enzyme will be prepared and cross-reaction patterns between soluble enzyme in various states of subunit interaction, and association with the membrane will be studied. Conversely antibody to membrane vesicles and partially purified enzyme derived from the membranes will be prepared and exhaustively absorbed with soluble enzyme that has been subjected to the entire purification procedure. The ability of the preabsorbed antisera to alter purine transport and/or membrane PRT'ase activity would serve as indication of other factors involved, in addition to the PRT'ase itself (as purified).

E. Genetic relationships. The genetic studies in progress outlined above will be continued in hopes of constructing a series of mutants for purine utilization which will be used as reagents for further study of mechanism and regulation of membrane plus enzymes in the uptake process. The genetic and enzyme analysis of such mutants with respect to membrane and soluble enzymatic activity, the ability of the soluble enzyme to associate with wild-type membranes and vice versa should provide insight into the genetic control of membrane-enzyme interaction, transport, and the variety of genetic events by which such control may be achieved. The variety of genetic mechanisms will be ascertained by appropriate mapping experiments using transduction in Salmonella and by use of a combination of transduction and conjugation in E. coli. The PRT'ase and uptake activities of a variety of appropriate strains carrying suitable genetic markers to act as recipient and/or donors of genetic material in such experiments have already been characterized in preparation for this line of experimentation.

F. Biosynthesis. The appearance of enzyme as soluble versus membrane bound will be studied utilizing a variety of techniques. Pulse-labeling of cellular protein will be employed as one approach. Determination of the specific activity of radioisotope in PRT'ase purified from membrane vesicles and from the pericytoplasmic space after subjecting intact cells to osmotic shock treatment should indicate whether the partitions that have been observed to date are random or whether they have a biological basis related to "age" or enzyme molecules. Another approach to the biogenesis of membrane enzymes involves enzyme induction. Since purine PRT'ases have been found to be induced significantly upon "forcing" cellular dependence on them, the appearance of membrane-associated PRT'ase activity under these conditions will also be studied by pulse labeling techniques. Attempts can then be made to correlate enzyme appearance with other concomitant biosynthetic processes in the membranes (e.g., phospholipid composition and turnover, assuming appropriate controls will be developed). Mutants requiring an unsaturated fatty acid such as those developed by R. Vagelos have been used to correlate membrane phospholipid synthesis with the appearance of sugar transport function and can be also used to study appearance of purine(pyrimidine) transport function.

Serial No. NHLI-131
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the β-Lysine Mutase Complex

Previous Serial Number: none

Principal Investigators: John J. Baker
Chris van der Drift

Other Investigator: T.C. Stadtman

Project Description:

Objectives: β-Lysine mutase is a complex enzyme system that consists of a cobamide protein of M.W. 160,000 and a smaller protein of M.W. 60,000. The reaction catalyzed by this complex is L-β-lysine $\rightleftharpoons$ L-erythro-3,5 diaminohexanoic acid. The cofactors required for activity of this complex are a B_{12} coenzyme, dithiothreitol, magnesium, a monovalent cation, ATP, and pyruvate. The roles of ATP and pyruvate in the action of this enzyme complex are not understood. Consequently the major objectives are to elucidate the role of these cofactors and to purify the protein components to homogeneity.

Major Findings:

β-Lysine mutase activity was measured by the acid ninhydrin procedure of Chinard. Since this assay is long and cannot readily measure initial rates, a spectrophotometric assay was developed by coupling the β-lysine mutase reaction with 3,5-diaminohexanoic acid dehydrogenase to form DPNH, 3-keto-5 aminohexanoic acid, and ammonia from 3,5 diaminohexanoic acid and DPN. The dehydrogenase was partially purified from the C. sticklandii extracts until free of DPNH oxidase activity. The purification steps were ammonium sulfate, G-25, DEAE cellulose and hydroxylapatite. In addition the components of the β-lysine mutase complex were purified from the same extract. The cobamide protein comes off the DEAE column about 50% pure. The smaller component was found in a different salt fraction and has been purified by DEAE cellulose chromatography. This smaller component catalyzes the activation of the cobamide protein by ATP. Some cobamide protein fractions give excellent initial rates in the spectrophotometric assay without ATP, but rapidly lose their ability to convert β-lysine to 3,5-diaminohexanoic acid unless ATP and the smaller protein component are present.

The role of pyruvate in the function of the enzyme complex is unknown. $NaBH_4$ (10^{-4} M), hydroxylamine (10^{-5} M), and phenylhydrazine (10^{-4} M) are potent inhibitors of β-lysine mutase. Preliminary experiments in which the enzyme was reduced with tritiated $NaBH_4$ followed by acid hydrolysis have

shown that several tritiated products were obtained. Unlike several other enzymes that contain electrophilic centers, neither lactate or alanine were products of the reduction.

Proposed Course of Action:

Future experiments will be concerned with the further purification of the protein components involved in the β-lysine mutase complex. In addition, the electrophilic center will be labeled with tritium and/or ^{14}C, the protein will be hydrolyzed by both acid and protolytic enzymes, and the electrophilic center identified. Experiments with ^{14}C and ^{32}P labeled ATP may determine why ATP is required for the enzyme to turnover.

Serial No. NHLI-132
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 1, 1971

Project Title: Genetics of E. coli Glutamine Synthetase

Principal Investigator: Mary Anne Berberich

Other investigators: none

Project Description:

objectives: Genetic studies on the glutamine synthetase enzyme system in E. coli have been initiated to further elucidate the relationships among the enzymes involved in nitrogen assimilation in this organism. Since the end products produced by each of the enzymes involved, as well as the concentration of inorganic nitrogen in the growth medium, influence the activity and, in some instances, the amount of enzyme produced, these studies were undertaken with a view toward determining whether there exists some regulatory system for this group of enzymes which operates at the genetic level.

Major Findings:

A strain of E. coli K_{12} which is prototrophic, streptomycin resistant and non-permissive was chosen as the parent strain. By a combination of mutagenesis with diethylsulfate and penicillin selection techniques, a group of mutants with desirable growth requirements have been isolated and this group is currently being characterized as to their enzymic defect.

Proposed Course of Research:

In particular, the relative positions of glutamine synthetase, glutaminase, glutamate dehydrogenase and glutamate synthetase on the E. coli chromosome will be studied by transduction and mating techniques. The first stage of this research project is currently in progress, namely, the selection of mutants minus or defective for the above enzymes to be used in mapping studies.

Attempts are also being made to screen for suitable specific-type inhibitors of glutamine synthetase which would make it possible to select for constitutive mutants. These types would be useful as well in this laboratory's biochemical work both for production purposes and for studies of protein structure.

It is hoped that enough mutants will be obtained for glutamine synthetase so that a fine structure genetic analysis may be carried out and compared with the biochemical evidence which suggests that the structural subunits of this protein are identical.

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Serial No. NHLI-133
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: A Dithiol Dehydrogenase of Clostridium sticklandii.

Principal Investigator: Lauren M. Cagen

Other Investigators: T.C. Stadtman

Cooperating Units: none

Project Description:

Objective: A dithiol dehydrogenase has recently been discovered in extracts of C. sticklandii. This enzyme differs from a previously reported mercaptan dehydrogenase in this organism in being specific for dithiol compounds. This enzyme will be purified and characterized.

Major Findings:

The reduction of tetrazolium dye by dialyzed extracts of C. sticklandii is greatly stimulated by FAD and by a dialyzable cofactor present in boiled extracts. FMN does not substitute for FAD. The enzyme will reduce added FAD in the absence of tetrazolium dye. The cofactor in boiled extracts does not seem to be a metal, but hasnot yet been identified. KCN also stimulates the reduction of tetrazolium dye. It has not been established whether this stimulation is due to chelation of an enzyme bound metal, discharge of an enzyme per sulfide group, or to some other activity of the cyenide. Preliminary purification experiments with DEAE cellulose, polycrylemide gel, and ammonium sulfate have been carried out.

Proposed Course of Action:

The enzyme will be further purified and characterization of the cofactor(s) in boiled extracts will be undertaken. The nature of the cyanide stimulation will be studied as well substrate specificity and ability of the enzyme to link DPNH oxidation to amino acid reduction by C. sticklandii extracts. It will be seen whether the dithiol dehydrogenase is distinct from the DPNH dehydrogenase also present in these extracts.

Part B No

Serial No. NHLI-134
1. Laboratory of Biochemistry
2. Section on Enzymes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) Methionine Biosynthesis and its Regulation in Fungi and Bacteria.
2) Mechanisms of Pyridoxal Phosphate Enzyme Catalyzed Elimination and Replacement Reactions.
3) Microbial Genetics of Microtubular Proteins and their Assembly.

Previous Serial Number: NHLI-198

Principal Investigators: Martin Flavin
Michael Savin

Other Investigators: Clarence Slaughter (technician)

Collaborating Units: J. Selhub and W. Sakami, Dept. of Biochemistry, Western Reserve Medical School; T. Fukasawa and K. Kurahashi, Institute for Protein Research, Osaka University

Project Description:

Objectives: Many amino acids have additional functions besides serving as building blocks for protein but methionine is conspicuous in this respect. It contributes to membrane phospholipid, in the form of the methyl groups of choline, as much as to protein, and in addition is a source of polyamines, a general methylating agent, via adenosylmethionine, and the initiator of protein synthesis. It would then not be surprising if the regulation of its synthesis were to reveal some unusual features. Previous work in this laboratory has shown that the synthesis of homocysteine, the immediate precursor of methionine, is accomplished in 3 sequential steps, which differ slightly in different microorganisms, and are catalyzed by: a) homoserine transacylase, utilizing succinyl CoA in Salmonella and acetyl CoA in Neurospora; b) cystathionine γ-synthase; and c) β-cystathionase. Our objective is to complete the characterization of these enzymes and determine the mechanisms by which their synthesis and activity is regulated. Cystathionine γ-synthase, which has been isolated in pure form from Salmonella, is a pyridoxal-P enzyme which catalyzes a unique spectrum of elimination and replacement reactions, and is being used to study the mechanisms of these reactions. A new objective is to obtain microbial mutants with alterations in the primary structure of their microtubular proteins, and eventually to study the structure and assembly of this organelle.

3KK

Major Findings:

Nine genes are known in Neurospora, mutation in which results in a nutritional requirement for methionine. As reported last year, extracts of mutants corresponding to 4 of these genes lack cystathionine synthase activity. Two of these mutants (me-3 and me-7) respond, as expected, to cystathionine as well as methionine. The others (me-1 and me-6) respond only to methionine and their extracts have additional deficiencies: me-1 lacks methylene tetrahydrofolate reductase activity, and me-6 lacks the folate polyglutamate derivatives needed for the non-B_{12} homocysteine transmethylase. The puzzling absence of cystathionine synthase activity from the latter 2 mutants has now been explained by the discovery that this enzyme activity is completely dependent on the presence of polyglutamate derivatives of N^5-methyltetrahydrofolate. This allosteric activation serves to prevent over-production of homocysteine in the absence of the methylfolate needed for its conversion to methionine; methylfolate also antagonizes the allosteric inhibition of cystathionine synthase by S-adenosylmethionine. It is noteworthy that in Neurospora it is the second enzyme of the homocysteine synthetic pathway that is subject to regulation.

Last year cystathionine synthase was reported to be absent from extracts of some species of Neurospora; these extracts have now been found to have activity when methylfolates are added. The intermediary role of cystathionine in methionine synthesis in yeast is still uncertain. We have now found significant cystathionine synthase activity in extracts of yeast protoplasts, but the reaction is not affected by addition of methylfolates and is much slower than the direct formation of homocysteine from acetylhomoserine and sulfide. The latter 2 reactions are catalyzed by the same enzyme, as in Salmonella and in contrast to Neurospora.

The regulation of homocysteine synthesis in Salmonella has been reinvestigated in relation to the questions: do methylfolates play any role, and can any clue be obtained as to why the first enzyme of the pathway appears to be uncontrolled in Neurospora. The results have not so far answered these questions. In Salmonella the first enzyme, homoserine trans-succinylase, is subject to synergistic end product inhibition by methionine + S-adenosylmethionine. It is of interest that the synergism is not observed in the absence of succinyl CoA, i.e. when the activity is assayed by an alternate reaction involving the exchange of labeled homoserine into succinylhomoserine. New procedures allowing precise measurement of the trans-succinylase in crude extracts have enabled us to reinvestigate the regulation of the synthesis of the 3 enzymes of the pathway. A mutant lacking S-adenosylmethionine synthase, scored as resistant to ethionine, was first identified in Neurospora in this laboratory. A similar E. coli mutant has been shown by others to be constitutive for the second and third enzymes, and we have shown that this is also the case for the first enzyme. The question of whether S-adenosylmethionine, or something derived from it, it also the corepressor for all 3 enzymes in

Salmonella is unresolved as we have not yet identified a mutant lacking S-adenosylmethionine synthase in this organism. Other results suggest that the mechanism regulating the synthesis of the first enzyme differs from that for the second and third in Salmonella. When the growth rate of a mutant which responds to either methionine or vitamine B_{12} is slowed in a chemostat by limiting the concentration of methionine the second and third enzymes are derepressed in a normal fashion, but it is virtually impossible to derepress the trans-succinylase. This result is not observed with vitamin B_{12} limitation, suggesting that exogenous methionine is a preferential source of a component involved in controlling the synthesis of the trans-succinylase. Two possibly related observations are that trans-succinylase activity is much reduced in stationary phase cells of wild type grown on minimal medium, and that the second and third enzymes are derepressed in mutants lacking the first.

In collaboration with colleagues at the University of Osaka, where the senior investigator spent 2 months as a guest of the Naito foundation, a study was begun of the role of S-adenosylmethionine in the processes of host DNA modification and restriction, utilizing bacterial mutants which have alterations in S-adenosylmethionine synthase.

The feasability of using fungi for genetic and chemical studies of microtubular proteins was explored. Colchicine binding protein was not detected in extracts of Neurospora or yeast. Colchicine and colcemid (10^{-3} M) did not prevent meiotic division in Neurospora. In preliminary experiments, the vegetative growth of Neurospora (wild type and a mutant with apparently generally increased permeability), Saccharomyces, and Schizosaccharomyces was not inhibited by: colchicine, colcemid, vinblastin, podophyllotoxin, or griseofulvin. In view of these results we have undertaken to aquaint ourselves with the genetics of Chlamydomonas, a microorganism with abundant extranuclear microtubules.

Proposed Course of Project:

The principle problems in homocysteine synthesis in Neurospora are to understand why it is the second enzyme in the pathway that is subject to metabolic control, and to elucidate the chemical nature of cystathionine synthase, in particular to determine whether the latter consists of a very weakly associated aggregate of 2 proteins coded by the me-3 and me-7 genes, one of which might have a purely regulatory function, or whether one of the proteins is cystathionine synthase and the other an enzyme which activates it, perhaps by methylation (allosteric inhibitor and activator are both reactive methylating reagents).

It may not be possible to pursue Project 2 during the coming year. There are 2 problems of particular interest at this stage (see last year's report by B. Posner). The capacity of cystathionine γ-synthase of Salmonella to catalyze the stereospecific or stereoselective exchange of α and β hydrogens in many amino acids, particularly diastereoisomeric amino acids, provides an opportunity to define the geometry of the active

site and the steric course of pyridoxal-P catalyzed reactions in general. The second relates to the observation that in the reaction succinylhomoserine → α-ketobutyrate there is partial direct transfer of both α and β hydrogen to carbon 4. A study of the conditions under which either the α or the β transfer will prevail over the other should illuminate the nature of the general base group(s) which attack these protons and the route by which the latter reach carbon 4.

We plan to search for Chlaymydomonas mutants in which either cell division or flagellar regeneration is resistant to inhibition by, or dependent on, colchicine and related substances. These mutants should have either altered apparatus for assembling microtubules or, hopefully, alterations in the structure of the colchicine binding protein. We hope also to devise improved methods for the assay and isolation of microtubular proteins through affinity labeling and chromatography.

Publications:

1. Kerr, D. S.: O-Acetylhomoserine and sulfhydrylase from Neurospora: Purification and consideration of its function in homocysteine and methionine synthesis. J. Biol. Chem. 246: 95-102, 1971.

2. Selhub, J., Savin, M. A., Sakami, W., and Flavin, M.: Synchronization of converging metabolic pathways: Activation of the cystathionine γ-synthase of Neurospora crassa by methyltetrahydrofolate. Proc. Nat. Acad. Sci. 68: 312-314, 1971.

3. Guggenheim, S. and Flavin, M.: Cystathionine γ-synthase from Salmonella: Spectral changes in the presence of substrates. J. Biol. Chem. (in press, June 1971).

4. Flavin, M.: Alternate pathways of methionine biosynthesis and their regulation. Tanabe symposium on amino acid metabolism (in press, 1971).

Abstracts:

1. M. A. Savin and M. Flavin: Regulation of homocysteine biosynthesis in Salmonella. Proc. Amer. Soc. Biol. Chem. (in press, May 1971).

Serial No. NHLI-135
1. Laboratory of Biochemistry
2. Section on Enzymes
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzyme Structure and Mechanisms of Action and Control

Previous Serial Number: NHLI-199

Principal Investigator: Ann Ginsburg

Other Investigators: S. Barbara Hennig
Carlos E. Caban
John B. Hunt
Mary Anne Berberich
Joseph E. Ciardi

Cooperating Units: P. D. Ross, D. M. Segal, and D. R. Davies, NIAMD

Project Description:

Objectives: 1) To study the physical and chemical properties of glutamine synthetase from Escherichia coli, particularly with respect to the correlation of the structure and catalytic function of this enzyme. 2) To study conformation and stabilization changes of a protein macromolecule effected through the specific binding of small molecules, and the relationship of such effects to enzyme catalysis and regulation. 3) To purify and study the ATP-glutamine synthetase adenylyltransferase from E. coli, with emphasis on the mechanism of action and the physical structure of this enzyme.

Major Findings:
1. Studies on the ATP: glutamine synthetase adenylyltransferase (ATase) from E. coli (Principal Investigators: S. B. Hennig, C. E. Caban, and J. E. Ciardi). Two active molecular forms of this enzyme were found: A relatively small protein of ~70,000 mol. wt. appears to be derived from a protein of ~130,000 mol. wt. during purification and storage at 4°. In collaborative studies with Drs. Wayne B. Anderson and E. R. Stadtman, it was shown by co-purification, polyacrylamide gel electrophoresis and heat-inactivation studies that the larger adenylyltransferase also possesses the deadenylylating activity (DA) attributable to the P_I-protein component of the DA two protein (P_I and P_{II}) system. In addition, the P_I-ATase, but not the smaller ATase, has appreciable L-glutamate (or L-glutamine) stimulated ATP-PP_i exchange activity. (The latter activity is presumably expressed through an ability of the ATase to catalyze a partial as well as a complete reversal reaction in the presence of Mg^{2+}, ATP, pyrophosphate, and allosteric activator.) In preliminary dissociation studies, the P_I-protein appeared to be composed of two subunits of unequal size. The inability of the small active ATase to catalyze either P_I-DA or ATP-PP_i exchange

activities has suggested that a subunit other than the small ATase in the P_I-protein complex is necessary for the expression of these activities. Whether or not the same catalytic site of the ATase when it is a part of the P_I-protein complex is involved in both the attachment and removal of AMP from glutamine synthetase (in adenylylation and deadenylylation reactions, respectively) is unknown. (It is known that specific metabolities have reciprocal effects on the adenylylation and deadenylylation reactions.) A physical characterization of the two active molecular forms of ATase is in progress.

2. On the binding of effectors to glutamine synthetase of E. coli.
(2a) The binding of feedback inhibitors to glutamine synthetase was further investigated by Dr. M. A. Berberich. The potential use of Sepharose-bound glutamine synthetase (catalytically active dodecameric aggregates) or of Sepharose-bound subunits (catalytically inactive) to study the interaction of effectors with this proteins was explored more thoroughly. Due to non-specific binding of all low-molecular weight compounds tested, it was concluded finally that columns of Sepharose-bound protein could not be used to study the specific binding of small molecules by the protein. Since Sepharose-4B itself does not bind effectors, the non-specific binding observed is a function of the Sepharose-protein matrix.

The binding characteristics of L-^{14}C-tryptophan in the presence of a high level of AMP (0.01 M) and, conversely, of ^{14}C-AMP in the presence of 0.01 M L-tryptophan to native glutamine synthetase was determined. The results show that there is an interaction between these allosteric inhibitor sites. Tryptophan at 10 mM concentration causes a 7-fold decrease in the affinity of glutamine synthetase for AMP, which binds to twelve apparently independent (non-interacting) sites. AMP at 10 mM, (but not at 1 mM) concentration causes a 3-fold decrease in affinity of the enzyme for L-tryptophan. Although the binding of L-tryptophan by glutamine synthetase remains cooperative with 10 mM AMP present, the number of L-tryptophan binding sites appears to increase to > 12/mole enzyme. The changes in the binding of L-tryptophan appear to be induced when glutamine synthetase is > 70% saturated, with AMP; only 8% saturation of the enzyme with L-tryptophan, however, significantly lowers the enzyme affinity for AMP.

(2b) A calorimetric study of the interaction of Mn^{2+} with glutamine synthetase was performed by Dr. J. B. Hunt in cooperation with Dr. P. D. Ross (NIAMD). These results show that 2 protons are displaced from the enzyme during the binding of each of the first twelve equivalents of Mn^{2+}. One proton is released instantaneously and the second proton is released in a slow process that is first order with respect to time and has a half time at 37° of 0.5-0.9 min or at 25° of 3-4 min. The slow thermal process may be attributed to a conformational change in the protein that is associated with an endothermic ΔH of $\sim +3$ kcal per mole subunit. The binding of Mn^{2+} to glutamine synthetase is largely entropy driven with $\Delta S \simeq +35$ cal/degree/mole subunit. Approximately two protons also are released from the enzyme for each equivalent of Mg^{2+} bound. The kinetics of the slow thermal process during the binding of Mn^{2+} (or Mg^{2+}) were similar to those

obtained by difference spectra measurements at 290.3 nm at either 37° or 25°. The first-order rate constants for the protein conformational change at 25° and 37° indicate that the heat of activation for this process is large ($\sim$ + 25 kcal per mole subunit). Despite the rather large perturbations in the tyrosyl and tryptophanyl spectral regions induced by the binding of Mn^{2+} (or Mg^{2+}) to glutamine synthetase, these cations, (as also either Co^{2+} or Zn^{2+}), have no detectable effect on the secondary or quaternary structure as determined by circular dichroism (CD) and optical rotatory dispersion (ORD) measurements (310-218 nm). From CD and ORD measurements, it was estimated that the α-helical content of native glutamine synthetase is $\sim$ 37%; 6 M guanidine·HCl converts the enzyme structure to 100% random coil.

3. X-Ray crystallography of glutamine synthetase from E. coli (D. M. Segal and D. R. Davies, NIAMD). Glutamine synthetase preparations in extreme adenylylation states (i.e. glutamine synthetase with one or twelve equivalents of 5'-adenylyl groups per mole enzyme) have been provided. The eventual growth of satisfactory crystals for X-ray analysis is anticipated.

Significance to Bio-Medical Research: The regulation and control of enzymic activities in vivo is of fundamental importance in cellular metabolism. Through studies in vitro these processes can be understood more fully. The study of structural changes that can be induced in a protein macromolecule are important in understanding cellular processes on a molecular basis.

Proposed Course of Project:

1. Studies of the binding of substrates and other effectors to glutamine synthetase of E. coli will be continued. In particular, allosteric linkage between different effectors of the enzyme will be investigated. Direct binding methods, measurement of proton release from the enzyme during metal binding by micro-pH methods, micro-calorimetric experiments (in collaboration with P. D. Ross, NIAMD), and possibly also NMR and epr studies will be employed in these studies.

2. Differential sedimentation techniques will be standardized using the Beckman Model E. ultracentrifuge in order to measure gross conformational changes induced in macromolecules by various effectors. Simultaneously induced micro-structural perturbations will be monitored by spectral, optical rotatory dispersion, and circular dichroic measurements.

3. Physical-chemical properties of the active molecular forms of ATP: glutamine synthetase adenylyltransferase will be determined by sedimentation-equilibrium, isoelectric focusing, electrophoresis, standardized Sephadex-gel filtration, and fluorescent techniques. Selective chemical modification of the sulfhydryl groups (or other amino acid residues) of this protein will be attempted in order to study structure-function relationships. Techniques to dissociate the P_I-adenylyltransferase will be explored for the purpose of studying the subunit structure and subunit reassociation.

4. Attempts will be made to isolate and characterize a 3'-5' cyclic AMP-dependent protein kinase from E. coli.

5. The X-ray crystallographic studies on glutamine synthetase by Drs. D. M. Segal and D. R. Davies (NIAMD) will continue.

Publications:

1. Stadtman, E. R., Ginsburg, A., Ciardi, J. E., Yeh, J., Hennig, S. B., and Shapiro, B. M.: Multiple molecular forms of glutamine synthetase produced by enzyme catalyzed adenylylation and deadenylylation reactions. Advan. Enz. Regulation 8: 99-118, 1970.

2. Ginsburg, A.: The preparation of 5'-^{14}C-adenylyl glutamine synthetase of Escherichia coli, H. Tabor and C. W. Tabor (eds.), Methods in Enzymology, Vol. XVII Part A, Academic Press, Inc., New York and London, 1970, p. 927-936.

3. Ginsburg, A. and Stadtman, E. R.: Multienzyme Systems. Ann. Review of Biochemistry 39: 429-472, 1970.

4. Deuel, T. F., Ginsburg, A., Yeh, J., Shelton, E., and Stadtman, E. R.: Bacillus subtilis glutamine synthetase: Purification and physical characterization. J. Biol. Chem. 245: 5195-5205, 1970.

5. Anderson, W. B., Hennig, S. B., Ginsburg, A., and Stadtman, E. R.: Association of ATP: Glutamine synthetase adenylyltransferase activity with the P_I component of the glutamine synthetase deadenylylation system. Proc. Natl. Acad. Sci. (U.S.) 67: 1417-1424, 1970.

6. Hennig, S. B., Anderson, W. B., and Ginsburg, A.: Adenosine triphosphate: Glutamine synthetase adenylyltransferase of Escherichia coli: Two active molecular Forms. Proc. Natl. Acad. Sci. (U.S.) 67: 1761-1768, 1970.

7. Hennig, S. B. and Ginsburg, A.: ATP: Glutamine synthetase adenylyltransferase from Escherichia coli: Purification and properties of a low-molecular weight enzyme form. Arch. Biochem. and Biophys., 1971 (in press).

Serial No. NHLI-136
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 1, 1971

Project Title: Zinc Induced Paracrystalline Aggregation of Glutamine Synthetase

Principal Investigator: Richard Miller

Other Investigators: E.R. Stadtman
P.Z. Smyrniotis

Project Description:

Objectives: 1) The effects of zinc upon native glutamine synthetase were studied using a kinetic spectrophotometric and spectrofluoromatic approach.

2) Purification and properties of glutamine-2-oxoglutarate aminotransferase, oxido reductase (glutamate synthetase).

Zinc induced spectral changes and zinc induced changes in TNS-enzyme fluorescence provides evidence for a marked zinc induced change in enzyme conformation.

Zinc may act either as an inhibitor or as an activator of glutamine synthetase biosynthetic activity depending upon pH, ATP concentration and Mg^{++} concentration. Furthermore, it has recently been shown that Zn^{++} can support γ -glutamyl transfer activity of unadenylylated glutamine synthetase.

Major Findings:

A previously unknown pathway for glutamate synthesis has been identified in Aerobacter aerogenes (tempest et al., Biochem. J. (1970) 117, 405).

glutamine + α-ketoglutarate + TPNH - TPN + 2, glutamate

The enzyme catalyzing this reaction has been purified to near homogeneity from crude extracts of E. coli W. $(NH_4)_2SO_4$ acetone, heat and gel filtration steps were employed in the purification. The protein gave a single symmetrical boundary in the analytical ultracentrifuge ($s_{20,w}$22S). The molecular weight (750,000) was determined by ultracentrifugation and gel filtration. As isolated the enzyme was associated with approximately one mole of flavin per 200,000 gms of protein. The bound flavin which consisted of both FMN and FAD was reduced by TPNH but not by DPNH.

The enzyme pH optimum was 7.8. The Km for α-ketoglutarate was 30 μM and for glutamine it was 170 μM. Preliminary gorwth experiments indicate that in batch cultures of E. coli w there is no relationship between the level of glutamate synthetase and that of glutamine synthetase, glutamate dehydrogenase or free NH_3. Preliminary electron micrographs indicate that glutamate synthetase is a large, multisubunit, spherical molecule.

Coupled with glutamine synthetase, (Km for NH_3 1.0 mM), glutamate synthetase has the capacity to play an important role in NH_3 fixation at levels of NH_3 far below those necessary for the function of glutamate dehydrogenase. Furthermore, the lack of correlation between levels of glutamate synthetase and those of glutamine synthetase, glutamate dehydrogenase and free NH_3 in batch cultures and its low Km's for substrates suggest that glutamate synthetase may be of major importance in NH_3 metabolism under all conditions.

Proposed Course of Action:

Studies are planned to elucidate further the kinetic and physical properties of glutamate synthetase.

Serial No. NHLI-137
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on L-asparaginase in anaerobic bacteria

Previous Serial Number 226

Principal Investigator: J.M. Poston

Other Investigators: none

Project Description:

Objectives: Certain neoplastic tissues have been shown to be adversely affected by invivo administration of L-asparaginase. This enzyme lowers the concentration of circulating L-asparagine causing the neoplastic tissues to involute and die while normal tissues are unaffected. The susceptible neoplastic tissues, unlike normal and unsusceptible neoplastic tissues, do not possess an adequate L-asparagine synthetase system. It was the objective of this study to isolate an organism which could serve as an alternative source of an L-asparaginase, especially one that was specific for L-asparagine and had no glutaminase activity. This would permit the study of the effect of lowering circulating L-asparagine without the complication of lowering the circulating glutamine as well.

Major Findings:

As was previously reported, an organism was isolated from soil by enrichment with L-asparagine and glycerol. It possesses L-asparaginase activity with an apparent K_m of 5×10^{-5} M and seems to have negligible glutaminase activity. This organism, strain 7Cl, grows well in a medium containing 1% L-asparagine, 1% glycerol, 0.06 M potassium phosphate (pH 7.0), trace metals, and made anaerobic by the addition of 0.03% Na_2S. It also grows well on sterile potato plugs either anaerobically or aerobically. Dr. Charles Zierdt of the Clinical Pathology Department of the Clinical Center, NIH, has tentatively identified 7Cl as a strain of Klebsiella pneumoniae. Although this is a facultative organism, 7Cl grows best under anaerobic conditions.

Extracts of the organism made by several methods were strongly active and retained their activity upon storage at -20° C for long periods. The activity is not affected by sulfhydryl reagents but there is marked inhibition by high concentrations (0.01 M) of magnesium, potassium EDTA.

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The activity has been refractory to purification. This seems to be due, at least in part, to increased lability upon purification. The activity is precipitated between 30 and 40 percent saturation with ammonium sulfate. It migrates in Sephadex G-150 columns as would be expected for a protein of about 90,000 daltons. Ion exchange has not shown any effect other than inactivation of the enzyme preparations.

Affinity chromatography effected striking purification but the lability of the activity was high and, as yet, no satisfactory method of stabilization has been found. Hexamethylene diamine was coupled to Sepharose 4B following cyanogen bromide treatment of the gel. d(-)-β-chloro-succinamic acid (prepared from L-asparagine by treatment with nitrous acid in the presence of sodium chloride) was coupled to the hexamethylene diamine side chain yielding, thereby, L-asparagine attached to the gel through a six-carbon spacer chain. Passage of crude extracts or partially purified fractions with from 6 to 60 fold purification. Specific activities of these preparations soon dropped to the original levels or lower.

Proposed Course of Action:

Pending availability of new methods of purification and stabilization, further work on this project has been terminated.

Part B No

Serial No. NHLI-138
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) Radioisotopic Assays for Glutamine Synthetase and Glutaminase.
2) Regulation of Clutaminase Activity in E. coli

Previous Serial Number: none

Principal Investigators: Stanley Prusiner

Other Investigators: E.R. Stadtman
L. Milner

Project Description:

Objectives: 1. To develop a sensitive and rapid assay for measurement of glutamine synthetase and glutaminase activities.

2. To study the regulation of glutaminase activity in E. coli.

Major Findings:

1. A very sensitive and rapid assay for glutamine synthetase and glutaminase has been developed by separating glutamate from glutamine on small columns containing dowex 1-Cl. The procedure has been shortened from several hours to a few minutes by the design of a vacuum manifold system which rapidly filters the sample over the ion exchange resin. The assay has also been extended for the measurement of asparagine synthetase and asparaginase activity.

2. Two glutaminases from E. coli have been reported in the literature previously with pH optima at 5 and 7. The relative amounts of these two enzymes vary markedly in the four strains of E. coli examined: ML, W, K-12, and B. Growth studies with E. coli B have shown that the pH 5 enzyme increases markedly during stationary phase while the pH 7 is highest during log and early stationary phase. These studies were complicated by the fact that the pH 7 enzyme is cold labile and both glutamine and glutamate have a profound activating effect on the catalytic activity of the pH 7 enzymes. Preliminary experiments suggest the pH 7 enzyme gives highest activity in crude extracts when the cells are CHO limited and have excess nitrogen available. The enzyme has been purified more than 1700 fold. Glutaminase requires glutamate and borate for stability at this stage of purification. The pH optima is 7 with almost no activity at pH 5. Substrate saturation studies show complex kinetics associated with cooperative phenomena.

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Proposed Course of Project:

1. Continue purification of glutaminase to homogeneity.

2. Physical and kinetic characterization of the purified enzyme.

3. Correlation of enzymic activity in crude extracts with NH_3 and glutamate levels of cells which can be varied depending on the growth conditions and stage of harvest.

4. Determine whether adenylylation system affects the activity of glutaminase.

5. Comparison properties pH 5 and 7 glutaminases.

6. Attempt to elucidate function of glutaminases by studying the properties of the enzyme in the isolated and crude forms and by genetic selection for mutations in the metabolism of glutamine.

Part B Yes

Publications

1. Prusiner, S. and Milner, L., A rapid radioactive assay for glutamine synthetase, glutaminase, asparagine synthetase, and asparaginase. Anal. Biochem. 37: 429-438, 1970.

2. Prusiner, S., Milner, L., Long, C.W., and Myers, M., Vacuum manifold for rapid assay of enzymes using radioactive tracers and ion exchange chromatography. Review of Scientific Methods, 1971 (in press).

Serial No. NHLI-139
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 1, 1971

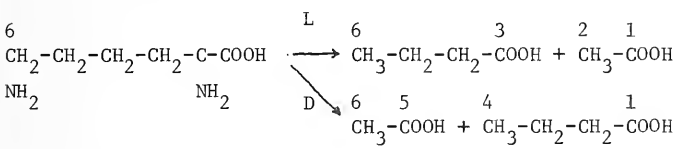
Project Title: Studies on Lysine Fermentation in Clostridia
The D-α-Lysine Pathway

Principal Investigator: J. Wai-Kuo Shih

Other Investigators: T.C. Stadtman

Project Description:

Objectives: It has been shown by this laboratory that Clostridium sticklandii converts DL-α-lysine to a mole each of acetate and butyrate and two moles of ammonia. The cell suspensions utilize both isomers of lysine as shown in Scheme I.



Scheme I

However, soluble enzyme preparations fail to produce acetate from carbons 5 and 6 of lysine (D-cleavage of Scheme I).

This investigator intends to study the metabolism of D-α-lysine in this organism and particularly:

(1) The conditions required for effective fermentation of D-α-lysine by cell free enzyme preparations.

(2) The systems involved in the utilization of 2,5-diamino hexanoate which is the product of D-α-lysine mutase. This reaction is thought to be the first step in the D-cleavage pathway.

Major Findings:

(1) For the differentiation of the L- and D- cleavages 6-C^{14}-DL- -lysine is used as substrate. Either D- or L- lysine can be used; presumably this organism contains lysine racimase in excess. The ratio of C^{14}-acetate to C^{14}-butyrate is a measure of the preference for the D-pathway. Wild-type and a few small-colony mutants of Clostridium sticklandii were examined.

Cell suspensions of small-colony mutant S3 and wild-type cultures grown on an arginine-lysine medium seemed capable of fermenting lysine by the D-cleavage more readily than regular cultures.

(2) For simplifying the tedious assay of C^{14} acetate and C^{14} butyrate as their hydroxmates, a revised method was employed. An acetokinase, specific for acetic acid was used to convert only acetate to the nonvolatile hydroxmate. The ratio of residual radioactivity after reaction with acetokinase and exhaustive evaporation to the total radioactivity in the original sample is a measure of the acetic acid content of incubation mixtures. Thus, C^{14}-acetate production from 6-C^{14} lysine is an assay of the D-lysine pathway.

Proposed Course of Research:

1. Conditions and cofactor requirements favorable for D-cleavage by the cell suspension system will be studied in more detail. Hopefully, the information gained can be applied to soluble enzyme preparations. It may be necessary to develop a revised technique of preparing cell free systems.

2. Both 1-C^{14} and 6-C^{14}-2,5-diamino hexanoate were prepared. These labeled materials will be applied to the study of the further metabolism of this compound and may help elucidate the defect in the metabolism of D-lysine by cell-free system.

Part B No

Serial No. NHLI-140
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1. Anaerobic metabolism of certain amino acids and other nitrogen compounds with especial reference to the role of B_{12} coenzyme and to electron transfer and phosphorylation reactions involved.

2. Methane biosynthesis and one-carbon compound metabolism.

Principal Investigator: T.C.Stadtman

Other Investigators: Colin G.D.Morley (Visiting Scientist, terminated Sept. 30, 1970) D-α-lysine mutase studies,
David Turner (NIH Postdoctoral Fellow, terminated Feb. 3, 1971) Glycine reductase studies.
H.Fu Kung (Visiting Scientist, terminated, Oct. 16, 1970). Nicotinic acid metabolism
James Shih (Visiting Scientist, see individual report)
Chris van der Drift (Visiting Scientist, see individual report)
John Baker (Postdocotral Fellow, see individual report)
Laren Cagen (Postodoctoral Fellow, see individual report)
Joseph N. Davis (Technical assistant and anaerobic Laboratory operator)
Jay Jones (Technical assistant).

Cooperating units: Dr. Lin Tsai (Lab.Biochem.,Section on Enzymes, NHLI)
Dr. J. Retey (Zurich, Switzerland)

Project Description:

Objectives: 1. Determination of mechanism of B_{12} coenzyme dependent amino group migration reactions of the lysine fermentation. (a) Purification and characterization of D-α-lysine mutase were carried out jointly with Dr. Colin Morley. Continuation of studies on further metabolism of D-α-lysine and 2,5-diaminohexanoate carried out by Dr. James Shih in cooperation with Dr. Lin Tsai.

(b) Purification and characterization of β-lysine mutase were continued by Dr. John Baker and Dr. Chris van der Drift. Investigation of roles of ATP and pyruvate are in progress.

2. Metabolism of nicotinic acid..characterization of the B_{12} coenzyme dependent step of the fermentation, and a subsequent isomerization of methylitaconate to dimethylmaleate was carried out by Dr. Hsiang-fu Kung in cooperation with Dr. Lin Tsai.

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3. Nature of electron transport and phosphorylation process linked to glycine reduction in Clostridium sticklandii. Purification of components of the multienzyme system by Dr. David Turner. Purification of flavoprotein electron transport protein by Dr. Laren Cagen.

4. Characterization of the menadione-dependent p-nitrophenyl-phosphatase of C. sticklandii. Experimental work carried out by Joe N. Davis.

5. Isolation and identification of acidic peptide growth factor required by wild type C. sticklandii for growth. Experimental work carried out by Jay Jones.

6. Mechanism of methane biosynthesis from formate by Methanococcus vannielii and from acetate by Methanosarcina barkeri.

Major Findings:

1. Lysine fermentation reactions:

In addition to its participation in the overall amino group migration reaction catalyzed by D-α-lysine mutase, the cobamide protein moiety of the enzyme complex catalyzes a slow pyridoxal phosphate and Mg^{++} dependent exchange of hydrogen between water and the C-6-methylene group of D-lysine. Several lines of evidence suggest that this exchange reaction and the migration of the amino group to the adjacent carbon atom are related processes and that both require Schiffs base formation between the terminal amino group of lysine and pyridoxal phosphate. Inhibition of the cobamide protein moiety by treatment with tetranitro-methane and by N-acetylimidazole, reagents known to modify tyrosine groups of proteins, suggests that as in certain other pyridoxal phosphate dependent enzymes, a tyrosine may be important for activity of the cobamide protein moiety of D-α-lysine mutase.

Although the intact D-α-lysine mutase complex appears to require ATP only as an allosteric activator and is equally stimulated by the phosphonic acid analogues of ATP, certain preparations of the separated protein components are active only with ATP. Separated components of β-lysine mutase behave in the same manner and it is tentatively concluded that ATP is additionally required in reaction(s) involving chemical modification of both of these mutases.

2. Studies of Dr. Kung established the obligatory intermediary roles of α-methyleneglutarate mutase (B_{12} coenzyme dependent) and methylitaconate isomerase in the overall fermentation of nicotinic acid to acetic and propionic acids and ammonia by Clostridium barkeri. The expected hydrogen migration reactions could be demonstrated by tritium and deuterium studies. Both enzymes were obtained in highly purified form and were considerably characterized.

3. Dr. Turner further purified to homogeniety the small acidic sulfhydryl protein moiety of the glycine reductase system of Clostridium sticklandii and also succeeded in further separation of the enzyme system into two highly purified somewhat larger protein fractions. The three recombined proteins still exhibited absolute requirement for phosphate in order to reduce glycine to acetate and ammonia. Thus extensive purification seems not to have caused loss of the concommitant phosphorylation reaction. Marked sensitivity to low levels of hydroxylamine suggests a carbonyl cofactor requirement for the reaction.

4. The pure menadione-dependent and SH^- dependent alkaline phosphatase of C. sticklandii which utilizes p-nitrophenyl phosphate as its only known substrate, failed to be labeled with P^{32} labeled substrate under a variety of experimental conditions. Current attempts to determin e the nature of the phosphorylated intermediate that finally is hydrolyzed to orthophosphate involve synthesis of the monophosphate ester of menadiol (reduced menadione). Utilization of this quinol monophosphate would further suggest a role of the phosphatase in an electron transport phosphorylation process.

5. The acidic peptide growth factor required by C. sticklandii has been highly purified from tryptic digests of cesein. Several active peptide fractions which contain 5 to 7 amino acid residues per peptide (as judged by gel filtration studies) have been isolated. The major amino acids in the active peptide fractions so far analyzed are: glutamic acid, valine, leucine, isoleucine, alanine and some glycine, serine and threonine. All other amino acids present in casein thus seem to be excluded as necessary components of the required peptide growth factor. The minimum composition of the active peptide(s) is not yet known.

6. Methane biosynthesis from formate by extracts of Methanococcus vannielii depends on ATP and hydrogen and is stimulated by various one-carbon derivatives of uracil and also by phosphoribosyl pyrophosphate. When ^{14}C-formate is used as substrate, a highly radioactive nucleotide fraction can be isolated from the bacterial extracts. This labeled material which is anionic, also serves as substrate for ^{14}C-methane synthesis. Experiments are in progress to characterize this material chemically and to determine whether it is an actual intermediate in the overall reduction pathway.

Proposed Course of Research:

Since all of the problems mentioned above except the study of nicotinate metabolism (2) are currently in progress, the general direction of the research outlined in each instance is that to be further pursued in the months to come.

Part B

Publications

1. T.C. Stadtman, "Vitamin B_{12}" , Science 171 859 (1971)

2. C.G.D. Morley and T.C. Stadtman, Studies on the Fermentation of D-α-lysine. PUrification and properties of an adenosinetriphosphate regulated B_{12} coenzyme dependent D-α-lysine mutase complex from Clostridium sticklandii, Biochemistry 9 4890 (1970).

3. C.G.D. Morley and T.C. Stadtman, STudies on the Fermentation of D-α-lysine. On the Hydrogen Shift Catalyzed by the B_{12} coenzyme Dependent D-α-lysine Mutase, Biochemistry (in press)

4. C.G.D. Morley and T.C. Stadtman, Studies on the Fermentation of D-α-lysine On the role of pyridoxal phosphate in the B_{12} coenzyme dependent D-α-lysine mutase reaction. (in preparation).

5. H.F. Kung and T.C. Stadtman, Nicotinic Acid Metabolism. VI. Purification and Properties of α-methyleneglutarate Mutase (B_{12}-dependent) and Methylitaconate Isomerase, J. Biol. Chem. 246:3746 (1971).

6. H.F. Kung, L. Tsai and T.C. Stadtman, Nicotinic Acid Metabolism. VIII. Tracer Studies on the Intermediary Roles of α-methyleneglutarate, Methylitaconate, Dimethylmaleate and Pyruvate. (in preparation).

7. T.C. Stadtman, "B_{12} coenzyme dependent amino group migrations" The Enzymes, Vol VI Ed. by P.D.Boyer, Academic Press (in press).

8. A Schwartz and T.C. Stadtman, Small Colonies of Clostridium sticklandii Resulting from Nitrosoguaindine Treatment and Exhibiting Defects in Catabolic Enzymes, J. Bact. 104, 1242 (1970).

Serial No. NHLI-141
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1. Effects of Divalent Cations and Energy Charge Upon the Activity of Glutamine Synthetase

Principal Investigator: Amiel Segal

Other Investigator: E.R. Stadtman

Project Description:

Objective: 1. The differential effects of Co^{++}, Mn^{++}, Mg^{++}, Ca^{++} and Cd^{++} upon the pH optimum, Km for substrates, catalytic activity and UV spectrum of glutamine synthetase indicate that each provokes a unique conformation of the enzyme. Cadmium, calcium and manganese salts inhibit the Mg^{++} dependent activity of unadenylated glutamine synthetase, whereas Co^{++} is an activator of the enzyme.

Like Mg^{++}, Co^{++} supports activity only of unadenylylated subunits, but the pH optimum feedback inhibitability, and dependence upon the metal/ATP ratio are similar to those of Mn^{++} dependent activity of adenylylated enzyme. With Mg^{++} or Mn^{++} the enzyme exhibits hyperbolic saturation functions for glutamate and NH_4 but Co^{++} dependent activity exhibits heterotropic and homotropic interactions with these substrates. The non linear relationship between specific activity and state of adenylylation supports the conclusion that heterologous interactions occur between adenylylated and unadenylylated subunits in hybrid molecules. The data suggest that adenylylation of a subunit abolishes its activity and decreases activity of adjacent unadenylylated subunits. These heterologous interactions render the co^{++} enzyme exquisitely sensitive to regulation by adenylylation and deadenylylation.

2. Mg^{++} dependent activity of unadenylylated glutamine synthetase is a function of the energy charge of the reaction mixture, whereas Mn^{++} dependent activity of adenylylated enzyme is considerably less dependent upon the energy charge. The response of the enzyme to energy charge is a function of the Mn^{++} concentration, with increasing sensitivity to energy charge at higher Mn^{++} concentrations. The inhibitability of glutamine synthetase by glycine and alanine increases markedly with increasing energy charge. These observations are consistent with similar observations made on other biosynthetic enzymes.

Proposed Course of Research: Temperarily discontinued.

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Serial No. NHLI-142
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Antibodies to E. coli Glutamine Synthetase

Previous Serial No. NHLI-208

Principal Investigator: Steven R. Tronick

Other Investigators: E.R. Stadtman
J.E. Ciardi

Project Description:

Objectives: 1) To characterize rabbit antibodies to adenylylated and unadenylylated E. coli glutamine synthetase.
2) To determine if these antibodies react with crude glutamine synthetase preparations from various microorganisms.
3) To determine if cross-reacting enzymes are modified by adenylylation.

Major Findings:

1) Complement fixation- The sensitive micro-complement fixation technique of Van Vanukis and Levine (Methods in Enzymol. 11, 928) was used in order to detect differences in the conformation of adenylylated (E_{12}) and unadenylylated (E_0) enzymes. This method was also useful in detecting classes of antibodies directed to different antigenic determinants on the enzymes. No differences were found between E_0 and E_{12} using antisera to either form of the enzyme. This result indicates that E_0 and E_{12} have very similar conformations. The peak of complement fixation (varying enzyme against constant antiserum) was broad-indicating many antigenic determinants on the enzymes. 0.007 ug/ml of enzyme was readily detected with a 1:20,000 dilution of antiserum. The following results suggest that adenylylation introduces a distinct antigenic determinant on the enzyme and its subunits. Antiserum to E_{12} fixed complement with E_{12}-subunits but not with E_0-subunits. An antiserum to E_{12} subunits fixed complement with adenylylated, but not unadenylylated subunits. This antiserum fixed complement with E_{12} (1/5 the amount fixed with E_{12} subunits) but not with E_0. Another antiserum to adenylylated subunits also fixed complement with E_{12}-subunits but not with E_0-subunits. (However, this antiserum fixed complement with E_0 and E_{12}: The titer for E_0 and E_{12} decreased during the course of immunization, while the titer for E_{12}-subunits greatly increased). The antisera to E_{12} and E_{12}-subunits did not bind AMP as measured by equilibrium dialysis, ammonium sulfate precipitation of Ab-Ag complexes, and precipitation of Ab-Ag complexes with anti-rabbit gamma globulin. In order to further characterize antibody

classes in each antiserum, competition experiments were performed. Although difficulties were encountered in obtaining reproducible data, the results suggest that subunits bind to E_{12} and E_0 antisera. Also, E_0-subunits appeared to bind to E_{12}-subunit antiserum. (Thus, separation of E_0- and E_{12}-subunits may be very difficult).

Complement fixation was also used to determine whether pure glutamine synthetases from other organisms are antigenically related to E. coli. Pseudomonas putida glutamine synthetase, obtained from Dr. A. Segal, partially cross-reacted (0.1 µg of enzyme fixed 65% less complement than 0.1 µg of E. coli enzyme). Bacillus subtillis glutamine synthetase, prepared by Dr. T.Deuel, did not cross-react. These results are in accord with those previously reported (No. 208, 1970) using the method of Ouchterlony.

2) Inactivation of enzyme activity- Gamma globulin purified from antisera to E_0 and E_{12} inhibited the γ-glutamyl transferase activity of E_0 and E_{12}. E_{12} was inhibited, by either antibody, to a greater extent than was E_0. Both E_0 and E_{12} were activated (130%) by an antiserum to E_{12}-subunits. Preliminary experiments indicated that the state of adenylylation of E_0 or E_{12} was not changed by the interaction with antibody.

3) Reaction with other glutamine synthetases- Crude extracts from fourteen microorganisms were examined for glutamine synthetase activity (γ-glutamyl transfer), for precipitin line formation with E_0 and E_{12} antisera in the Ouchterlony assay, and for inhibition of transferase activity by anti-E_0 and E_{12} gamma globulin. Methanosarcina barkerii, Clostridium sticklandii, Neurospora crassa, Bacillus subtilis, and Bacillus licheniformis did not exhibit transferase activity nor did they form precipitin lines in the Ouchterlony assay. Acanthamoeba castellanii, Streptomyces rutgersensis, and Streptomyces diastachromogenes extracts displayed transferase activity but were not inhibited by high levels of antibody. They did not form precipitin bands. Salmonella typhimurium, Klebsiella pneumoniae, and Proteus mirabilis crude extracts catalyzed the γ-glutamyl transfer reaction and formed precipitin bands which fused with the precipitin bands of pure and crude E. coli glutamine synthetase (indicating antigenic homology). The transferase activity in these extracts was inhibited by antibody, under standard conditions, to about the same extend (25%) as the E. coli enzyme. The transferase activity in Pseudomonas putida crude extracts from cells grown on 4 mM NH_4^+ (unadenylylated enzyme) was not inhibited by antibody; however, the activity in crude extracts of cells grown on 40 mM NH_4^+ (adenylylated enzyme) was inhibited by 50%. Both types of extracts formed precipitin lines which spurred with the E. coli, S. typhimurium, and K. pneumoniae bands (indicating partial antigenic homology).

Surprisingly, crude Azotobacter vinelandii extracts formed precipitin lines with antibody. Transferase activity supported by Mg^{++} was inhibited by 70% with a standard concentration of E. coli antibody. The Mn^{++} supported activity was inhibited by only 30% as was the activity without added metal ion. NOrmal gamma globulin had no effect on any of these activities.

Proposed Course of Action:

The various crude glutamine synthetases that cross-react with antibody to the E. coli enzyme have all been derived from gram-negative organisms. Several more gram-negative and gram-positive organisms will be assayed in order to determine if, in general, the glutamine synthetases from gram-negative organisms are antigenically related (to E. coli). Attempts will be made to determine if the cross-reacting enzymes are adenylylated (as shown directly by Dr. A. Segal for the P. putida enzyme and indirectly by Gancedo and Holzer, (Europ. J. Biochem., 4, 190, 1968) for some enteric bacteria).

The enzyme from A. vinelandii will be studied in more detail because of its fascinating dependence on Mg^{++} for reaction with antibody and its intriguing activation by metal ions.

The E. coli antisera will be further characterized using complement fixation in order to resolve subunit and "AMP"-specific classes of antibodies. The inhibition of transferase activity by antibody as a function of state of adenylylation will be examined in more detail. The effect of antibody on biosynthetic activity will also be studied.

Part B no.

Serial No. NHLI-143
Laboratory of Biochemistry
Section on Enzymes
Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: 1) Application of Physical Methods in the Determination of Structures of Organic Compounds.
2) Synthetic Studies of Organic Compounds of Biological Interest.

Previous Series No. 135

Principal Investigator: L. Tsai

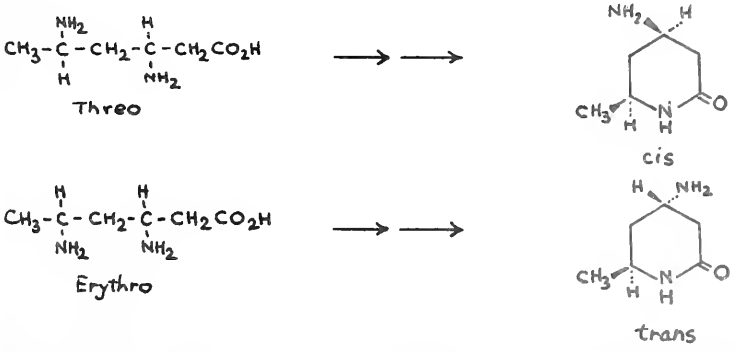
Other Investigator: E. Caveney (technician)

Project Description:

Objective (1) Stereochemistry of 3,5-diaminohexanoic acid contains two asymmetric centers (C-3 and C-5) giving rise to two pairs of enantiomers which are present in the synthetic material obtained from the action of ammonia on sorbic acid. These isomers can be recognized as a high-melting and a low-melting one. Enzymatic studies conducted by T.C. Stadtman indicated activity for the low-melting isomer. Since the second asymmetric center (C-5) is generated by the course of the enzymatic reaction, the knowledge of the stereochemistry of the isomers of 3,5-diaminohexanoic acid would be of significant value to the understanding of the enzymatic reaction.

Major Findings:

The stereochemical relationship of the isomeric open-chain amino acids and the cyclized derivatives is shown as follows (the figure shows only one enantiomer of each pair):



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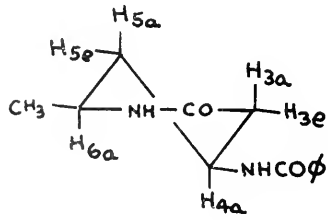
Thus the configuration of the amino acid can be deduced from the knowledge of the stereochemistry of the aminolactam. The NMR spectra of the benzoylated derivatives of the aminolactam derived from the isomeric amino acids were examined. By means of decoupling technique and the preparation of a dideuterated compound from the high-melting isomer the following set of parameters were obtained for each spectrum:

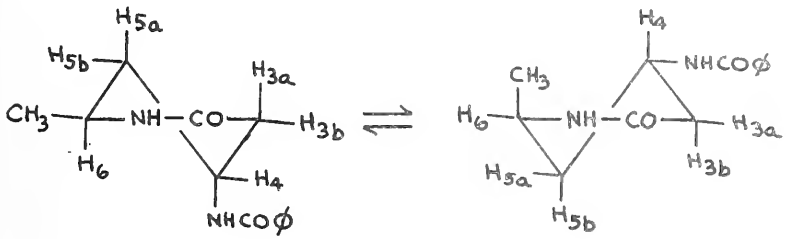
High-melting isomer				Low-melting isomer			
	(ppm)(CD_3OD)		J (Hz)		(ppm)(CD_3OD)		J(Hz)
CH_3	1.231	CH_3-H_{6a}	6.5	CH_2	1.245	CH_3-H_6	6.5
H_{6a}	3.58	H_{6a}-H_{5a}	12	H_6	3.69	H_6-H_{5a}	7
H_{5a}	1.38	H_{6a}-H_{5e}	3	H_{5a}	1.74	H_6-H_{5b}	6
H_{5e}	2.15	H_{5a}-H_{5e}	-13	H_{5b}	2.065	H_{5a}-H_{5b}	14
H_{4a}	4.35	H_{4a}-H_{5a}	12	H_4	4.46	H_4-H_{5a}	3
H_{3a}	2.27	H_{4a}-H_{5e}	3	H_{3a}	2.38	H_4-H_{5b}	8
H_{3e}	2.605	H_{4a}-H_{3a}	11	H_{3b}	2.65	H_4-H_{3a}	7
		H_{3a}-H_{3e}	-18			H_4-H_{3b}	6
		H_{3e}-H_{5e}	- 2			H_{3a}-H_{3b}	18
		H_{4a}-H_{3e}	6				

These assignments were finally confirmed by the good agreement between the measured and the computed spectra obtained by a "Spin Simulation Program". According to the Karplus relationship between the vicinal coupling constants and the dihedral angle of the vicinal protons, the three large values (11-12 Hz) in the spectrum of the high-melting isomer can be attributed to interaction of di-axial protons and the three small ones (3 Hz) to those of axial-equitorial protons. To accommodate such an arrangement the methyl group at C-6 and the amino group at C-4 in the aminolactam must have a cis relationship and the preferred conformation for the molecule would be the one with the two substituents in equitorial position in a twist chair form (see figure).



High-melting Isomer

The spectrum of the low-melting isomer, on the other hand, has all six vicinal coupling constants in relatively small values (3-8 H_z). This can be interpreted as a spectrum of a trans isomers whose two substituents (methyl group at C-6 and amino group at C-4) are necessarily situated in axial and equitorial positions. Thus there is no one preferred conformation for this molecule, and instead, it probably exists in solution at room temperature as an equilibrium mixture of the two possible conformers (see Figure). The observed spectrum is therefore a time-averaged one.



Low-melting Isomer

The trans arrangement for the aminolactam leads to an erythroconfiguration for the low-melting isomer of the amino acid. Since the enzymatically active isomer of β-lysine is known to have a S-configuration, the new asymmetric center, C-5, in 3,5-diaminohexanoic acid must therefore have a S-configuration.

Proposed Course of Action:

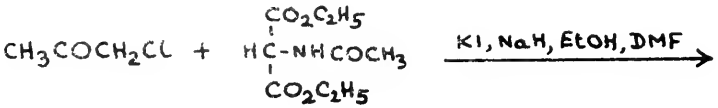
The stereochemistry of 2,5-aminohexanoic acid, intermediate in α-lysine metabolism, shall be studied by chemical and physical methods.

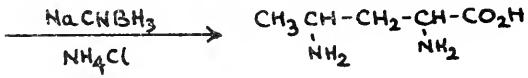
Project Description (2)

2,4-diaminopentanoic acid: 2,4-diaminopentoic acid has been recently characterized as an intermediate in the ornithine metabolism (Freedmann et al. 1970 and Dyer and Costilow, 1970) by C. sticklandii. Since this amino acid has not been synthesized previously, we have undertaken to develop a practical synthesis.

Major Findings: (2)

A synthesis of 2,4-diaminopentanoic acid was accomplished as outlined below:





The structure of the product was in good agreement with the NMR spectrum.

Proposed Course of Action:

Optimum conditions for each step of the above synthesis as well as methods for purification of the product shall be studied in detail.

Part B Yes

Publications

1. H.F. Kung, S. Cederbaum, L. Tsai and T.C. Stadtman, Nicotinic Acid Metabolism. V. A Cobamide Coenzyme. Dependent Conversion of α-Methyleneglutaric Acid to Dimethylmaleic Acid, Proc. Natl. Acad. Sci., U.S., 65, 978 (1970).

Serial No.: NHLI-144
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enzymatic fragmentation of fibrinogen.

Previous Serial No.: None

Principal Investigators: Elemer Mihalyi
David Towne

Project Description:

Objectives: To determine the mode of formation and nature of heavy fragments of fibrinogen during proteolysis.

Methods Employed and Major Findings:

pH-Stat, Sephadex column chromatography, sedimentation equilibrium.

It was shown previously that trypsin splits the fibrinogen molecule into 3 fragments of approximately 90,000 mol. wt. (Mihalyi and Godfrey). Investigation of its fragmentation by plasmin (Marder et al., Fletcher et al.) showed a number of heavier intermediates than the one described above, but the reaction mechanism was not elucidated. Therefore, the digestion process with trypsin was re-investigated using exclusion column chromatography (Sephadex G200) to isolate the high molecular weight fragments. The elution diagrams of serially inhibited digests were resolved with a computer program (written by R.I. Shrager of DCRT, NIH) into the individual components. The digestion process itself was monitored by the pH-stat, and each sample was defined by the number of peptide bonds split per mol of fibrinogen. A total of 6 species were identified including the native fibrinogen. Samples of these, at various stages of the digestion, were obtained from the Sephadex column and their molecular weights estimated by the Yphantis' high speed equilibrium ultracentrifugation method. All the species above 90,000 mol. wt. show self association and non-ideality, which make the estimation of molecular weights uncertain. However, they appear homogeneous and their properties remain constant during the digestion. If the digestion is not pursued beyond 80% of the bonds split in the "slow reaction" the products are: 3 fragments of 83,000 mol. wt., 2 of 16,000 mol. wt. and the remaining mass, corresponding to approximately 20% of the original molecule, appears as small peptides. The fragments preceding these have approximate molecular weights of 240,000 and 150,000 and they build up and disappear during the digestion. Their kinetics indicates a rapid conversion of the native molecule into the 240,000 species, with simultaneous liberation of the 16,000 mol. wt. fragments and of most of the peptide material. What is left is essentially the three still interconnected 83,000 and 150,000 mol.

wt. fragments. The latter is then further split into the two 83,000 fragments. The whole kinetic scheme can be reproduced with remarkable accuracy by the computer. The 83,000 fragments appear to be further degraded, although at a very slow rate, whereas the 16,000 fragments appear to be final products.

All these intermediates were missed when the analytical ultracentrifuge was used to monitor the process. Clearly, for analysis of mixtures, the zonal methods, like the Sephadex gel filtration, and also SDS gel electrophoresis, are more advantageous.

Signigicance to Bio-Medical Research: To determine in a precise manner, in vitro, the events occurring during the process of thrombolysis.

Proposed Course of Research: Completed.

Publications:
None.

Serial No. NHLI-145
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Isolation and characterization of cellular growth factors from calf serum.

Previous Serial No.: None

Principal Investigator: F.H. White, Jr.

Other Investigators: Charles D. Scher (NCI)
Allen Rein (Bionetics, Inc., Bethesda, Md.)
Gilbert Wright (Technical)

Project Description:

Objectives: To isolate and study growth factors from calf serum in regard to their effects (e.g. on mitotis, cellular migration, thymidine incorporation, and protein synthesis) upon normal and virally transformed mammalian cells in culture.

Methods employed:

1. Ethanol fractionation, ammonium sulfate fractionation, and ion exchange chromatography have been employed in the purification of a growth factor from calf serum.

2. Assay of growth factor is based upon mitotic effects and uptake of tritiated thymidine by mouse fibroblast cells.

Major findings:

A fifty fold purification has been achieved on a factor from calf serum which increases the rate of mitosis and thymidine uptake in cell culture.

Significance to Biomedical Research: A better understanding of the mitotic process would have obvious fundamental as well as clinical significance and may be approached by isolation and study of the factors that influence it.

Proposed Course of Research: A continued purification of growth factor(s) from serum will be undertaken, followed by characterization. The possibility of tritiation of the purified factor(s) and radioautographic study in cell cultures will be pursued.

Publications:
None.

Serial No.: NHLI-146
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of conformation on the free radical distributions of irradiated proteins.

Previous Serial No.: NHLI-216, 217, 218

Principal Investigator: F.H. White, Jr.

Other Investigators:
T. Smitherman (NCI)
P. Riesz (NCI)
H. Moss (NCI, Technical)
G. Wright (Technical)

Project Description:

Objectives: 1) To determine distributions of free radicals throughout the individual residues of an irradiated protein and to correlate with the secondary and tertiary structures. 2) To study the isotope effect between hydrogen and tritium as it affects the labeling process.

Methods Employed:

1. Electron spin Resonance (E.S.R.) has been employed for semi-quantitative determination of free radical content in irradiated proteins.

2. Proteins were tritiated by the "free radical interceptor" method (White, et al., Radiation Res. 32, 744 (1967)); alternatively, "radiation-induced hydrogen transfer" (RIHT) was used (P. Riesz, Radiation Res. 44, 35 (1970)). This process involved incorporation of tritium onto exchangeable sites, followed by γ-irradiation, whereupon a fraction of the tritium becomes incorporated onto non-exchangeable sites by an unknown pathway.

3. Denaturation of tritiated proteins was achieved by reduction and carboxymethylation. The products were subjected to chymotryptic digestion; peptides were isolated by ion exchange chromatography and electrophoresis.

4. Amino acid analysis in conjunction with scintillation flow counting was employed as recently described (F.H. White and C. Mencken, Anal. Biochem. 34, 470 (1968)).

Major Findings:

1. It has already been shown that the distribution of free radicals in

an irradiated protein is influenced by conformational characteristics of the protein (F.H. White, Radiation Res. 36, 470 (1968)). In a continuation of this study, lysozyme has been labeled by the "RIHT" process, denatured, and digested proteolytically. The peptides have been separated, and analyses are in progress to determine the extent of labeling of individual residues.

2. The validity of this approach depends upon the following hypothesis: that the distribution of tritium among the amino acids reflects the distribution of carbon free-radicals. Evidence for this hypothesis has been summarized (P. Riesz and F.H. White, Jr., Advances in Chem. Series 81, I, p. 496 (1968)).

In a continuing investigation of this hypothesis, the isotope effect between hydrogen and tritium has been studied. It has been determined, with the use of tritiated deuterium sulfide as the labeling reagent, in comparison to HST, that the isotope effect is approximately 2. With this factor taken inot consideration, there is still an approximate equivalence between free radical content (as estimated by E.S.R.) and tritium content. Demonstration of this equivalence is essential for validity of the above hypothesis.

Significance to Biomedical Research: This work has been undertaken to clarify the relationship between the conformational features of proteins and radiolysis. Thus further insight may be obtained into the effects of ionizing radiation upon the living organism, and the possible use of the above labeling techniques as methods for investigation of protein conformation may be explored.

Proposed Course of Research: The investigation will be continued to examine further the specific activities of individual amino acids in tritium-labeled proteins and to correlate these activities with their conformational features.

Publications:

Riesz, P. and White, F.H., Jr.: Radical distributions in γ-irradiated dry proteins at 195°K. Radiation Res. 44: 24, 1970.

White, F.H., Jr., Kon, H., and Riesz, P.: Comparison of electrical discharge with γ-radiation for the production of free radicals in lyophilized proteins. Radiation Res. 45: 8, 1971.

White, F.H., Jr. and Mencken, C.: Construction of a pressure-resistant flow cell of high efficiency for scintillation counting with accelerated amino acid analysis. Anal. Biochem. 34: 560, 1970.

White, F.H., Jr.: Thiolation of Proteins , in revised Vol. XI, Methods in Enzymology, in press.

White, F.H., Jr.: Reduction and Reoxidation at Disulfide Bonds , in revised Vol. XI, Methods in Enzymology, in press.

Miller, J.H., White, F.H., Jr., Riesz, P., and Kon, H.: Distribution of free radicals among amino acids from lyophilized UV-irradiated proteins. Photochem. and Photobiol., accepted for publication.

Serial No. NHLI-147
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the tritiation of a peptide derived from nisin.

Previous Serial No.: None

Principal Investigator: F.H. White, Jr.

Other Investigators: E. Gross(NICHHD)
G. Wright (Technical)

Project Description:

Objectives: To employ a peptide, derived from the bacterial antibiotic nisin, as a model compound in the investigation of side reactions that proceed during the tritiation of proteins with tritiated hydrogen sulfide (HST).

Methods Employed:

1. Tritium labeling was achieved by the free radical interceptor method, with the aid of electrical discharge (White, et al., Anal. Biochem. 30, 295 (1969)), followed by exposure to HST. Evidence has accumulated (P. Riesz and F.H. White, Jr., Advances in Chem. Series 81, Vol. I, p. 496 (1968)) that the resulting distribution of tritium among the amino acid residues reflects the distribution of free radicals from exposure to electrical discharge or ionizing radiation.

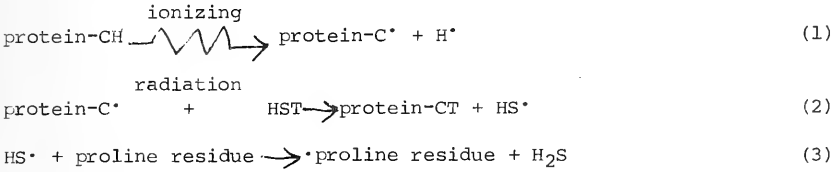
2. Amino acid analysis with scintillation flow counting (F.H. White and C. Mencken, Anal. Biochem. 34, 560 (1970)) was employed to determine distribution of tritium among amino acids of hydrolysates of the labeled peptide.

3. Purification of nisin, proteolytic cleavage to yield a tridecapeptide, and saturation of the dehydroalanine within this peptide, were carried out as described by E. Gross and J. Morell (Proc. Nat. Acad. Sci. 62, 952 (1969)). Attachment of proline residues to the peptide was achieved by activation of the carboxyl group with NN'-carbonyl diimidazole.

Major findings:

There are three unique structural features of the nisin peptide. 1) It contains no proline; 2) it contains no cystine or cysteine; and 3) it contains dehydroalanine, an amino acid rarely found in proteins.

The nisin peptide has been prolinated and subjected to comparative studies with the original peptide to determine whether the presence proline, covalently bound, (3 residues per molecule of peptide) would cause an increase in the tritium content or alter the tritium distribution, or both. The following reactions may be involved:



Reaction (1) indicates the formation of a free radical on carbon as a result of exposure of the protein to ionizing radiation. By reaction (2), tritium is incorporated into the protein and HS• is liberated. This product is partially eliminated as shown by reaction (3), whereby HS• abstracts a hydrogen atom from a weak C-H bond, known to exist within the proline ring, to yield a free radical on proline. The latter radical migrates to other positions within the protein, and reacts further with HST to perpetuate a chain reaction.

Amino acid analyses of nisin peptide and prolylnisin peptide indicate the following:

1. Average specific activity of nisin peptide = 0.69 μCi/mg.
2. Average specific activity of prolylnisin peptide = 1.9
3. Tritium distributions of these two peptides were not significantly different.

Conclusions: The presence of prolyl residues raises the tritium content by approximately a factor of 3, and therefore the chain reaction described above proceeds to a significant extent. However, the effects of prolyl residues on the distribution of tritium among the other amino acid residues were negligible and therefore the chain reaction does not detract from this method as an indicator of free radical distributions.

Significance to Biomedical Research: Because of the rising use of radioisotopes for medical purposes and the increasing radiation hazards throughout our society, it is becoming increasingly necessary to understand the mechanisms involved in radiation damage. Since free radicals play a significant role in radiation damage, emphasis has been placed upon the study of their distributions in proteins and peptides. Therefore the method of studying these distributions, developed in this laboratory, has been subjected to continued scrutiny to determine its advantages and limitations. The information so obtained would also be relevant to application of the present technique to the tritium-labeling of proteins for use in biological tracer studies.

Proposed Course of Research: The project will be extended to examine the effects of introduction of disulfide bonds by reaction of the unsaturated residue in nisin peptide with hydrogen sulfide, followed by oxidation.

Publications:
None.

Serial No. NHLI-148
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biological oxidation capabilities of membrane fragments obtained from E. coli.

Previous Serial No.: NHLI-219

Principal Investigator: Richard W. Hendler

Other Investigator: Amelia B. Yarbrough

Project Description:

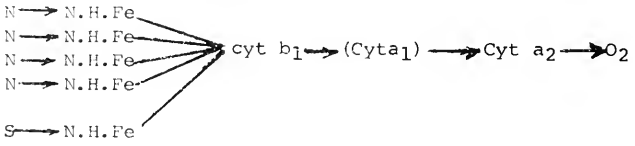
Objectives: The primary source of energy in respiring cells is a series of discrete oxidation reactions called electron transport. These reactions occur in cell membranes. My work in protein synthesis,as well as that of several other groups, has provided strong evidence that a primary site of of protein synthesis is at a membrane surface. Current ideas in protein synthesis suggest that energy produced from electron transport in a membrane is sent out into the aqueous milieu as ATP and eventually brought back to the membrane in the form of activated amino acids. We are interested in the possibility that these two major metabolic pathways, one exergonic and the other endergonic, may be directly coupled in the membrane. We are approaching this problem from two directions that we hope will eventually converge. This report concerns the studies of biological oxidation in E. coli membrane fragments.

Methods Employed and Major Findings:

A. The involvement of non-heme iron proteins in E. coli respiration. Our earlier studies indicated the participation of an electron carrier between succinate dehydrogenase and the cytochrome chain. The evidence was that a soluble cell factor plus bovine serum albumin could inhibit succinoxidase more severely than succinate dehydrogenase and at the same time have no effect on NADH oxidase. Since we have shown that in E. coli, succinate and NADH use the same cytochrome chain, the block must occur between succinate dehydrogenase and the chain. A non-heme iron protein is thought to occupy this position in mitochondrial electron transport chains. Preliminary evidence for the participation of non-heme iron proteins in both NADH and succinate oxidations was obtained by noting the sensitivity of these processes to four different metal chelators. In order to measure the kinetics of non-heme iron reduction by NADH and succinate, we devised a rapid sampling and freezing technique for electron paramagnetic resonance (EPR) spectroscopy. We found that in 5 seconds, NADH could reduce about 50% as much non-heme iron as could be reduced by dithionite and that in

15 to 20 seconds, 90 to 100% of the fully reduced signal could be obtained. This in relation to the observation that in 5 seconds about 70% of the cytochrome b_1 is reduced by NADH and the results of the metal chelator studies present strong evidence for the direct participation of non-heme iron in NADH oxidation by the electron transport chain of E. coli. The EPR signal elicited by succinate was very weak -- about 10-20% of the dithionite induced signal. Because of this, initial reduction rates could not be measured. The maximum succinate indiced signal is reached at about 30 to 40 seconds. This corresponds with the observation that the maximum level of cytochrome b_1 reduction by succinate also occurs at that time. These observations, plus the sensitivity of succinate oxidation to metal chelators argue for including a non-heme iron protein in the succinoxidase pathway. We have further established that the metal chelator works by preventing the reduction of non-heme iron (and also all of the cytochromes) but that the reduced chain can be readily reoxidized, in the presence of the chelator. The cell factor which inhibits succinoxidase (in the presence of serum albumin) resembled the metal chelator in that when it was present, cytochrome reduction by succinate was inhibited, but reoxidation of the reduced chain was not impaired. NADH reduction of the cytochrome chain in the presence of the cellular inhibitor was not affected.

B. Characterization of the electron transport chain.
Our studies indicate that the respiratory assembly in E. coli can be represented as:



N= NADH Dehydrogenase
S= Succinate dehydrogenase

The participation of cytochromes b_1, a_1, and a_2 in a single electron transport chain available to both NADH and succinate is shown by 1) the ability of either substrate to reduce almost all of each cytochrome; 2) the fact that the duration of the steady state is the same for all three cytochromes when either substrate is present; 3) the fact that when the cytochromes have been reduced by succinate, an oxygen pulse can oxidize 50 to 60% of each of the cytochromes in 6 milliseconds. The relative positions of cytochrome b_1 and cytochrome a_2 is indicated by the response to cyanide which inhibits the reduction of cytochrome a_2 more than cytochrome b_1, but which allows the reoxidation of a_2 more readily than b_1. The evidence for the inclusion of non-heme iron proteins was given in part A above. The reasons for including many NADH dehydrogenase-non-heme iron units in relation to succinate dehydrogenase-non-heme iron units are: 1) the non-heme iron EPR signal elicited by NADH is at least 5 times stronger than the one elicited by succinate. 2) NADH dehydrogenase activity

is at least 5 times greater than succinate dehydrogenase activity. 3) NADH oxidase activity is at least 5 times greater than succinoxidase activity.

C. Fractionation of the respiratory chain of E. coli.
We have studied conditions for solubilizing the membranes of E. coli and at the same time preserving enzymatic activity. The most popular solubilizer, sodium dodecyl sulfate completely destroyed oxidative activity. We have developed a procedure, using deoxycholate, (DOC), which leads to membrane solubilization and preservation of dehydrogenase activity. This procedure also solubilizes the cytochromes. Using DOC impregnated acrylamide gels for an electrophoretic fractionation, we can locate specific dehydrogenase bands, by employing the cytochemical redox stain, nitro blue tetrazolium. From the membranes we have obtained at least 4 succinate dehydrogenase bands (on the gel) and one NADH dehydrogenase band. The soluble part of the cell contains no succinate dehydrogenase but two faster (smaller molecular weight species) of NADH dehydrogenase are present. We have grown E. coli in the presence of Fe^{55} in order to label both heme and non-heme iron proteins. Using a column of Sepharose 4B it appears that we are able to separate the cytochromes from non-heme iron and also to resolve cytochrome b_1 from cytochromes a_1 and a_2. A separation of succinate dehydrogenase from NADH dehydrogenase was also achieved.

Significance to Bio-Medical Research: This project is designed to provide some understanding of the manner in which an organized cellular system may efficiently integrate complex metabolic pathways. The potential importance of biochemistry lies in working out chemical pathways and then considering this knowledge in terms of living cells and tissues. If it could be demonstrated that the structural organization of the cell contributes to its ability to integrate complex and related biochemical pathways, a better understanding of cellular function would result.

Proposed Course of Research: Full attention will be given to the separation and characterization of the individual components of the respiratory assembly of E. coli. Attempts for reconstitution of the chain from the separated components will be made.

Publications:

Hendler, R.W.: Respiration and protein synthesis in Escherichia coli membrane-envelope fragments. VI. On the reduction of non-heme iron and the cytochromes by NADH and succinate. J. Cell Biol. (submitted for publication).

Serial No. NHLI-149
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Location of Tritium in Tritiated Methonine.

Previous Serial No.: None

Principal Investigator: F.H. White, Jr.

Other Investigator: G. Wright (Technical)

Project Description:

Objectives: To study the radiolytic breakdown of methionine.

Methods employed:

1. Tritium labeling of proteins was achieved by the "free-radical interceptor" method (White, et al., Anal. Biochem. 30, 295 (1969)) which involves creation of free radicals by ionizing radiation or electrical discharge followed by reaction with tritiated hydrogen sulfide (HST).

2. To isolate tritiated methionine, acid hydrolysates of tritiated proteins were subjected to ion exchange chromatography.

3. Degradation of tritiated methionine was achieved by reaction with cyanogen bromide (CNBr), by oxidation with L-amino acid oxidase, or by reaction with acetic anhydride to form the azlactone. The first method is specific for the methyl group. The latter two methods give information on the extent to which tritium is located on the alpha carbon.

Major Findings:

1. The results with CNBr degradation indicate that approximately 30% of the tritium of methionine resides on the methyl group. This finding thus far appears independent of the kind of protein employed or whether it is native or denatured.

2. Reaction of native proteins by enzymatic oxidation or with acetic anhydride indicate none of the tritium on the alpha carbon. Location of the remaining tritium remains to be determined.

Significance to Biomedical Research: It is well known that methionine is one of the most radiolabile amino acids in proteins, and an understanding of its breakdown is essential to an understanding of radiation damage in general. It is well established that free radicals play an important role

in radiation damage, and the "free radical interceptor method" should give information on the location of radicals within the methionine of irradiated proteins; hence also on the relation of free radical distribution to radiolysis.

Proposed course of research: More data will be sought for methionine derived from various proteins, irradiated by various means. The project will be extended to other amino acids.

Publications:
None.

Serial No. NHLI-150
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Biosynthetic capabilities of membrane fragments obtained from E. coli.

Previous Serial No.: NHLI-221

Principal Investigators: Richard W. Hendler
Raymond Scharff

Collaborating Investigator for Electron Microscopic Studies:
N. Nanninga of the Laboratory for Electron Microscopie, University of Amsterdam, Netherlands.

Project Description:

Objectives: In the belief that complex metabolic processes in living cells depend on important spatial relations of the components, as maintained by the structural elements of the cell, we have developed procedures for isolating cellular membranes by very gentle techniques. These procedures as well as the chemical and cytological characterization of the membrane-envelope fragments were described in the last report (1970). We have determined that these fragments have high respiratory activity and are quite active in the synthesis of protein, RNA and DNA. There is currently great interest in the mechanism of DNA replication because the soluble enzyme (DNA polymerase) originally thought to carry out this process may be simply a repair enzyme. We have decided to concentrate attention on a particulate DNA-synthesizing activity that we have found in one of the membrane-envelope containing fractions (see report for 1970). The present report describes experiments which show that the particulate activity (P_3) is qualitatively distinct from the soluble activity (S_3) and that it does not represent simply the adsorption of the soluble enzyme.

Methods Employed and Major Findings:

A. Qualitative differences in particulate and soluble DNA-synthesizing activities.
1) P_3 responded better to more native DNA - The soluble DNA polymerase responds more to denatured than native DNA. Using commercial calf thymus DNA, which is already denatured, S_3 was stimulated equally by heated and unheated DNA. P_3 was stimulated 108% by unheated and only 48% by heated DNA.

2) ATP enhances the ability of P_3 but not S_3 to respond to added DNA - ATP (1mM) stimulated the activity of P_3 in the presence of heated DNA by

21% and in the presence of unheated DNA by 34%. ATP (1mM) did not stimulate the activity of S_3 in the presence of heated or unheated DNA.

3) P_3 appears to be more nearly saturated with endogenous DNA than does S_3. Added heated DNA stimulated S_3 by 865% but stimulated P_3 by only 48%.

4) The Mg^{++} optimum concentration for P_3 activity in the presence of ATP and unheated DNA was about 7mM; for S_3 it was about 12mM.

5) Deoxycholate at a concentration of 0.2% (diluted to 0.04% during the assay) inhibited S_3 activity by 70-80% but inhibited P_3 activity by only 23-38% (over a range of Mg^{++} concentrations from 2 to 12mM).

6) p-Chloromercuribenzoate stimulated endogenous S_3 activity several fold but did not effect endogenous P_3 activity.

B. Sedimentation characteristics of S_3 and P_3 DNA synthesizing activities.

The P_3 fraction contains only 0.7% of the total cellular soluble enzyme activity for DPNH-ferricyanide reductase and about 20% of the total DNA synthesizing activity. Therefore, if adsorption of the soluble enzyme is involved in the P_3 activity, it is a highly specific adsorption. A sucrose layer centrifugation system was worked out to separate soluble protein from membranes and ribosomes. The soluble fraction was identified by DPNH-ferricyanide reductase activity and by using bovine plasma albumin dyed with procion blue. Membranes were assayed by the enzyme activity, succinate dehydrogenase, and ribosomes by absorption at 260nm. Electron microscopy was also used to characterize the fractions. Using a 9% sucrose layer on top of a 30% sucrose layer, the soluble proteins stay on top, the ribosomes concentrate at the interface and the membranes concentrate in the pellet. Although this method gives a considerable degree of separation of membranes and ribosomes, electron microscopy shows some small membrane vesicles in the interface and some large ribosomal clusters in the pellet. Using the system, the following percent distribution of DNA-synthesizing activity was found:

	S_3	P_3
Soluble	92±5	38±4
Interface	11±9	46±4
Pellet	2.2±1.8	27±11

Preliminary experiments indicate that all of the fractions from P_3 and the interface fraction from S_3 are stimulated by ATP, but that the soluble fraction from S_3 is not stimulated. We have attempted to artifactually cause the S_3 activity to penetrate the sucrose layers to see if some of the sedimenting activity of P_3 was similarly induced. The addition of excess DNA (which complexes with DNA polymerase) to S_3 did not change the distribution of activity. The addition of P_3 fraction to S_3 did not change its sedimentation distribution. The addition of excess DNA plus

P_3 did not change the sedimentation distribution.

Significance to Bio-Medical Research: This project is designed to provide some understanding of the manner in which an organized cellular system may efficiently integrate complex metabolic pathways. The potential importance of biochemistry lies in working out chemical pathways and then considering this knowledge in terms of living cells and tissues. If it could be demonstrated that the structural organization of a cell contributes to its ability to integrate complex and related biochemical pathways, a better understanding of cellular function would result.

Proposed Course of Research:

1. We will continue to study the properties of the particulate DNA-synthesizing system in order to establish its identity as distinct from that of the soluble DNA polymerase.

2. We will try to further purify the cytological unit possessing the DNA-synthesizing activity.

3. Studies will be initiated with the E. coli mutant that lacks the DNA polymerase but which is capable of replicating DNA.

4. The DNA product synthesized by the P_3 fraction will be characterized to establish the nature of the replication and the fidelity of copying added DNA template.

Publications:

Hendler, R.W.: Biological Membrane Ultrastructure. Physiological Reviews 51: 1, 1971.

Serial No. NHLI-151
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The interaction of actin and myosin.

Previous Serial No.: None

Principal Investigators: Evan Eisenberg
W. Wayne Kielley

Other Investigator: Louis Dobkin

Project Description:

Objectives: It is now generally recognized that contraction of muscle involves the interaction of the two proteins actin and myosin with ATP. Clearly therefore it is of importance to determine the strength of binding and the binding ratio of actin to myosin both in the presence and absence of ATP. This is difficult to accomplish with myosin because it occurs as heterogeneous filaments at low ionic strength but heavy meromyosin (HMM), a tryptic digestion product of myosin, is monomeric under these conditions and therefore its binding to actin can be studied using the analytical ultracentrifuge.

In the present study we investigated the binding of HMM to actin in the absence of ATP under varied conditions of ionic strength and temperature, and compared this binding with that observed in the presence of ATP as determined from kinetic measurements.

Methods Employed and Major Findings:

The binding of actin to heavy meromyosin (HMM) was studied using a Beckman Model E analytical ultracentrifuge equipped with photoelectric scanning absorption optics. Actin was purified by Sephadex G-200 column chromatography. At 0.1M KCl, 1mM Mg, 10mM imidazole pH 7, 25° ca. 1.8μM actin was found to be non-sedimentable regardless of the initial actin concentration which ranged from 2.3 to 46.0μM. Thus a "critical" concentration of G-actin was apparently present. With 5mM P_i present this critical concentration was reduced to ca. 0.7μM. When increasing amounts of HMM were added to 10μM actin under these conditions a maximum of one mole of HMM was found to bind 2 moles of actin monomer. This same binding ratio was observed in the absence of P_i at 20mM KCl, at 4°, and when the added actin concentration was 4μM. Furthermore under all these conditions the binding constant was too high to measure -- greater than $2X10^6$M. This binding is many-fold stronger than the binding determined in the presence of ATP from kinetic measurements. Furthermore preliminary binding studies in the presence of

ATP have been performed using the analytical ultracentrifuge which lead to the intriguing possibility that in the steady state a fraction of the HMM is always dissociated from the actin, no matter how high the actin concentration is. This suggests that during the cycle of actin-HMM interaction, much of the HMM exists in a "refractory" form in which it cannot bind to actin. If this is in fact the case it may have significant implications for the situation in vivo. The flexability of contracting muscle as opposed to rigor muscle may occur because in the rigor state all of the myosin bridges are bound to the actin filament whereas in contracting muscle only a fraction of these bridges are bound. In support of this hypothesis X-ray diffraction studies of contracting muscle show that less than 50% of the myosin bridges are closely associated with actin. Therefore the occurrence of a refractory period during which the myosin molecule cannot bind to actin may play a key role in the cyclic interaction of actin and myosin which causes contraction in vivo.

In addition to work on this project, we have also been involved in isolating the individual components of the native tropomyosin system which is involved in the relaxation of skeletal muscle. Using column chromatography on hydroxyapetite and DEAE Sephadex, we have presently isolated four components of this system in a relatively homogeneous form as shown by gel electrophoresis.

Significance to Bio-Medical Research: This work is aimed at gaining a better understanding of the basic mechanisms of muscle motility, a phenomenon which occurs not only in skeletal muscle, but also in such diverse systems as cardiac muscle, arterial smooth muscle, platelets, and perhaps within all cells where protoplasmic streaming occurs.

Proposed Course of Research: We plan to continue our investigation of the binding of actin to HMM in the presence of ATP using both the analytical ultracentrifuge and kinetic methods. We also plan to investigate the effect of PP_i and ADP on this binding. Finally we plan to investigate the effect of the purified native tropomyosin components on the interaction of actin and HMM both in the presence and absence of ATP.

Publications:

Eisenberg, E. and Kielley, W.W.: Native tropomyosin: Effect on the interaction of actin with heavy meromyosin and subfragment-1. Biochem. Biophys. Res. Comm. 40: 1, 1970.

Eisenberg, E. and Weihing, R.: Effect of skeletal muscle native tropomyosin on the interaction of amoeba actin with heavy meromyosin. Nature 228: 1092, 1970.

Serial No.: NHLI-152
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure of the muscle protein, myosin.

Previous Serial No.: NHLI-222

Principal Investigators: W. Wayne Kielley
Juanita Cooke

Other Investigator: Louis Dobkin

Project Description:

Objectives: The continuing objectives of this program are identification of the details of molecular structure as they relate to the biochemical properties of the protein and its function in muscle contraction.

The myosin molecule is composed of two identical polypeptide chains each of 200,000 molecular weight. In addition about 21% of the mass of all native myosin preparations consists of smaller polypeptide chains of about 20,000 molecular weight. It has not been possible, so far, to separate the latter material from the large chains except under denaturing conditions. While the small chains are widely regarded as a functional part of the myosin molecule, this has not been proved. No stoichiometry in the structure has been demonstrated, a problem that is complicated by the fact that this low molecular weight fraction is composed of four components in unequal amounts. Conflicting figures in the literature are probably confused by the fact that myosin prepared by "standard" laboratory procedures always contains 5-10% of low molecular weight contaminants readily removable by any one of three chromatographic procedures. Some time ago, before the presence of the small chains in myosin was recognized it was observed in this laboratory that myosin possessed histidine as an amino terminal group. Subsequently, another laboratory reported the presence of a blocked N-terminal, identified as acetyl serine. The past year has been devoted to developing methods of separating the low and high molecular weight chains without resorting to denaturing conditions and end group analysis of the low and high molecular weight chains.

Methods Employed and Major Findings:

End group analysis has employed both enzymatic (pronase digestion) and chemical (dinitrophenylation) methods. It has now been determined that the large chains of myosin possess the amino terminal histidine, while the small chain fraction possesses one component, readily separable from the rest by chromatography, which contains a blocked amino terminal,

tentatively identified as acetyl serine. In addition the small chain fraction possesses amino terminal alanine, about six-tenths of a mole per mole of small chains, and a minor amount, about 0.1 mole, of either aspartic or glutamic acid. Disc gel electrophoresis of the small chains show the presence of two major and two minor components. With the high recovery of N-terminal alanine it must be assumed that two of the four components have amino terminal alanine and the other two recognized N-terminal structures account for the other two components.

Significance to Bio-Medical Research: This work is aimed at a better understanding of the mechanism of muscular contraction.

Proposed Course of Research: We will continue to try to define the role of the low molecular weight chains in the activity of myosin and to further characterize them. Further work on the amino acid sequence of functional areas (ATPase, actin binding) of the molecule is also planned.

Publications:

None.

Serial No.: NHLI-153
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Plasmin Digestion of Fibrinogen.

Previous Serial No.: None

Principal Investigators: Robert M. Weinberg
Elemer Mihalyi

Other Investigators: None

Project Description:

Objectives: 1. To determine the kinetics of the proteolysis of fibrinogen by plasmin; 2. To investigate the structural modifications of the fibrinogen molecule during the course of this reaction.

Methods Employed and Major Findings:

Kinetics determined by pH Stat. Plasmin assay (TAME) in pH Stat. Sephadex G-200 column chromatography. SDS disc gel electrophoresis. Ultraviolet gel scanning. Computer programs for analysis of kinetic data and simulation of plasmin-fibrinogen proteolysis.

Kinetic analysis of the plasmin-fibrinogen reaction reveals 2-3 bonds split at a fast rate and 54 bonds split at a rate 15-20 slower. Rupture of this number of bonds is sufficient to produce the "final" reaction products, although the reaction proceeds very slowly for several more hours. The splitting of bonds was correlated with production of heavy intermediates in sequentially inhibited digestion mixtures. The complete sequence of events was rationalized in terms of the three nodule model of fibrinogen. After a fast initial modification of the molecule, resulting in a loss of about 20% of its original mass the enzyme splits one of the two connecting segments of the nodules. This results in the appearance of a species with two modules and a final conversion to single nodules. One-third of the latter is further degraded to a lower molecular weight product. This model has been used as the basis of a computer simulation of the digestion and accords a close fit to experimental data.

Significance to Bio-Medical Research: To determine in a precise manner, in vitro, the events occurring during the process of thrombolysis.

Proposed Course of Research: To isolate and further characterize the intermediate and final fibrinogen digestion products.

Publications: None

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Serial No.: NHLI-154
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The structure and biochemical activity of actin and myosin.

Previous Serial No.: NHLI-214

Principal Investigators: Robert Adelstein
W. Michael Kuehl
W. Wayne Kielley

Other Investigator: Mary Anne Conti

Project Description:

Objectives: The 17 cyanogen bromide peptides comprising the entire actin molecule (MW = 45,000) have been isolated and identified as to amino acid composition and NH_2 terminal residues (Adelstein and Kuehl, Biochemistry 9, 1355, 1970). With one exception the 17 peptides have been placed in sequence. Although the missing overlap can be inferred, experiments are presently under way to identify it, thus concluding this phase of the work.

Methods Employed and Major Findings:

The cyanogen bromide peptides were placed in order by isolating and uniquely identifying the methionine containing tryptic peptides. Both column chromatography and peptide mapping on paper were employed.

In working with the large, insoluble cyanogen bromide peptides the classical techniques in peptide separation and purification have had to be modified. The recently developed technique of maleating the ϵ -NH_2 group of lysine in order to produce only C-terminal arginine peptides on tryptic digestion has been used to complete the ordering of the cyanogen bromide peptides. Moreover maleation had the added benefit of solubilizing some of the larger peptides.

To date we have placed all but one of the 17 cyanogen bromide peptides in order. This information together with the sequence of each of the individual cyanogen bromide peptides being carried out by Dr. M. Elzinga at the Retina Foundation in Boston will yield the entire sequence of the 406 residues of rabbit skeletal actin.

Actin has been shown to be a single polypeptide chain with a uniquely placed residue of 3-methylhistidine in the 3rd cyanogen bromide peptide (starting from the NH_2 terminal end). Unlike myosin it does not contain

methylated lysines. (On the other hand, certain myosins in this laboratory have been found not to contain 3-methylhistidine).

Significance to Bio-Medical Research: This work is aimed at a better understanding of muscular contraction. The ultimate aim of these structural studies is to pinpoint those amino acid-residues (methylated as well as non-methylated) that play a critical role in contraction and those residues which if either altered (e.g. demethylated) or replaced, would result in muscle disease.

Similarities between actins isolated from a number of species (e.g. ameba) indicates that this work on the primary structure of rabbit skeletal actin should be applicable to actins from other sources.

Proposed course of Research: After the cyanogen bromide fragments of rabbit skeletal actin have been ordered studies will be undertaken to elucidate the active site of the actin molecule (e.g. that part which interacts with myosin). Preliminary studies have been undertaken to identify a unique sulfhydryl in one of the six half cysteine residues that might be involved in actin:actin or actin:myosin interactions.

Publications:

Kuehl, W.M. and Adelstein, R.S.: The absence of 3-methylhistidine in red, cardiac and fetal myosin. Biochem. Biophys. Res. Comm. 39, 5, 1970.

Serial No.: NHLI-155
1. Laboratory of Biochemistry
2. Cellular Physiology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The isolation and purification of human platelet myosin.

Previous Serial No.: None

Principal Investigators: Robert Adelstein
W. Michael Kuehl
W. Wayne Kielley

Other Investigator: Mary Anne Conti.

Project Description:

Objectives: The presence of myosin and actin-like proteins in blood platelets have been reported in other laboratories. However these proteins have never been well purified nor have their properties been defined.

It is the purpose of this study to isolate and purify the myosin-like protein in human blood platelets and to compare these properties to myosin isolated from skeletal and smooth muscle. Moreover the role of the muscle-like proteins in platelet contraction and the possible role in thrombus formation will be studied.

Methods Employed and Major Findings:

In isolating a myosin-like protein from blood platelets we have made use of classical methods of protein isolation (i.e. high ionic strength extraction with low ionic strength precipitation, $(NH_4)_2SO_4$ fractionation) as well as more recently developed techniques (Sepharose fractionation, affinity binding of platelet myosin to rabbit skeletal actin). Other techniques used include electron microscopy, SDS polyacrylamide gelelectrophoresis, and amino acid analysis. This last technique has been modified to include the identification of methylated amino acids that have been found in this (Kuehl and Adelstein, BBRC, 371, 1969) and other laboratories to exist in contractile proteins from numerous sources (e.g. ameba actin and platelet actin).

Starting with platelet concentrates we have succeeded in isolating two myosin like proteins. Both proteins resemble smooth muscle myosin in having ATPase enzymic activity that is stimulated by both EDTA and Ca^{++} and inhibited by Mg^{++}. Moreover both proteins bind to rabbit skeletal actin and are released by Mg-ATP.

The two proteins differ in molecular weight, the heavier on coelectrophoresing with the heavy (200,000) subunit of rabbit skeletal myosin in 0.1% SDS polyacrylamide gel electrophoresis. The lighter protein has a molecular weight of α100,000 in the same system.

Electronmicroscopic confirmation of structural similarities to rabbit skeletal myosin has recently been obtained with Dr. Thomas Pollard. The heavier species forms aggregates in the presence of low salt which are similar to the "thick filaments" observed for skeletal myosin. Moreover the lighter species forms "arrowheads" on interaction with rabbit skeletal actin - similar to rabbit skeletal heavy meromyosin.

Significance to Bio-Medical Research: The purpose of isolating contractile proteins from platelets is to discover the role that these proteins play in platelet aggregation, platelet contraction and possible thrombus formation. Furthermore it now appears that there are marked similarities between muscle and platelet myosin and therefore any understanding of how platelet myosin works should give insight into the mechanism of muscle contraction.

Proposed Course of Research: The exact relationship between the heavy and light myosin-like proteins will be studied. The light species may represent a proteolytic subfragment of the heavier molecule, which is similar to the proteolytic fragment of skeletal myosin in having retained both actin binding and enzymic properties of the parent molecule. It is possible that the light fragments may have physiological significance, being generated by thrombin digestion.

An effort to isolate and purify the actin-like protein of platelets will be undertaken and future studies will involve the interaction of these two contractile proteins and their relationship to thrombus formation and clot retraction.

Publications:

None.

Serial No. NHLI-156
1. Laboratory of Biochemistry
2. Cellular Biochemistry and Ultrastructure
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Membrane Biochemistry

Previous Serial No.: NHLI-223

Principal Investigators: Edward D. Korn
Andrew G. Ulsamer
Thomas Olivecrona
Edward Victoria

Other Investigators: Paddy L. Wright

Project Description:

Objectives: To study the chemical, physical, structural and metabolic properties of plasma membranes.

Methods Employed: Plasma membranes are isolated from the amoeba, Acanthamoeba castellanii, by sucrose gradient centrifugation and subjected to the usual chemical analyses for lipids, proteins, carbohydrates, and enzymes. Membranes are dissolved in appropriate solvents and proteins and carbohydrates fractionated by gel chromatography and analysed by polyacrylamide gel electrophoresis. In metabolic experiments the usual radioactive tracer techniques are employed.

Major Findings:
1. Isolation of plasma membranes from the amoebae has become a routine procedure enabling the preparation of 30-50 mg of membranes of high purity. It has been found that plasma membranes from young cells (5 day cultures) contain an alkaline phosphatase of high specific activity which is purified some 15-20 fold over the whole homogenate. Possibly a Mg-ATPase, but no other enzyme has yet been found associated with the plasma membrane (except for 5'-nucleotidase activity which may be due to the alkaline phosphatase).

This low enzymatic content relative to plasma membranes of mammalian and bacterial cells is not entirely unexpected because there is no evidence to suggest that the amoeba is capable of active transport and other metabolic activities associated with the plasma membrane of other cell types.

2. The isolated plasma membrane can be extracted with chloroform;methanol to give a lipid-free residue (the lipids have been characterized previously). This residue is completely soluble in 5% sodium dodecylsulfate or in 8M urea. It contains a high concentration of non-lipid phosphorus (1 μmole/mg protein)

and carbohydrate (anthrone reaction; 1 μmole "glucose"/mg protein). When a urea solution of the lipid-free residue is chromatographed on Sephadex-G 200, the P and carbohydrate are quantitatively recovered in a voided peak together with a small fraction of the protein. Most of the protein is eluted near the salt boundary with an apparent molecular weight less than 15,000. Indeed, much of the protein is too small to be voided on G-25 and none of it is voided on G-50. SDS-gel electrophoresis of the original membrane, or of the lipid-free residue gives at the most 3 protein bands (which also stain for carbohydrate) each of which migrates in the region where standard proteins of less than 15,000 molecular weight are found.

The phosphorus is not released as inorganic phosphorus upon hydrolysis in 3N HCl at 100° for several hours. The nature of the residues to which it is linked is not known.

The carbohydrate contains glucose, xylose, mannose and at least one unknown sugar.

Significance to Bio-Medical Research: An understanding of the structure, function and biosynthesis of the plasma membrane is necessary if we are to understand such important processes as transport, cell-cell interactions, pinocytosis and phagocytosis among others.

Proposed Course of Project: It is hoped to determine the number of protein constituents in the plasma membrane and to define their general properties. It is also intended to identify the nature of the non-lipid phosphorylated compound. In addition, the effect of selective removal of membrane lipids on the membrane structure will be studied by spectroscopic, enzymatic and electron microscopic techniques.

Serial No. NHLI-157
1. Laboratory of Biochemistry
2. Cellular Biochemistry and Ultrastructure
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Cytology of Acanthamoeba

Previous Serial No.: NHLI-225

Principal Investigators: Blair Bowers
Edward D. Korn

Other Investigators: Thomas E. Olszewski
Robert J. Anstead

Cooperating Units: NASA, Quality Assurance Division

Project Description:

Objectives: To elucidate the structural basis of biochemical and physiological events in the soil ameba, Acanthamoeba castellanii. Our current emphasis is on the study of pinocytosis and phagocytosis in the ameba.

Methods Employed:
1. Transmission electron microscopy: standard techniques of fixation, embedding, and sectioning are the main techniques employed.

2. Light microscopy: phase contrast and Nomarski interference optics are used to examine living cells for correlation with the electron microscopic images.

3. Radioactive tracers are being used for quantitative studies of uptake of soluble molecules by Acanthamoeba.

4. In collaboration with Robert J. Anstead, the scanning electron microscope is being used for examination of whole cells at magnification and resolution higher than that obtainable with the light microscope.

Major Findings:
1. Our major effort this year has been in the quantitative study of pinocytosis in the ameba in order to assess its significance in surface membrane turnover. With the soluble protein, horse radish peroxidase, it is possible to visualize pinocytosis with the electron microscope. This evidence has indicated that the soluble molecules enter the cell by small surface vesiculations of 100-200 mμ in diameter. Using scintillation counting of tritiated compounds to assess uptake of other molecules, we have studied the uptake

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process of inulin, serum albumin, glucose and amino acids by the ameba. Each of these quite different compounds appears to be taken up at approximately the same rate. For albumin the uptake is linear over a wide concentration range. The biochemical data are consistent with the EM observations and point to pinocytosis as the major, if not exclusive, mechanism for uptake of soluble molecules from the medium. Pinocytosis appears to occur continuously in the culture medium. The quantitative data for uptake together with the EM observations of the mode of uptake allow an estimate of surface depletion by pinocytosis. Assuming a pinocytic vesicle size of 100 mμ and an average ameba surface of 2200 μ^2, then the observed rate of pinocytosis would result in complete surface turnover roughly every minute.

2. To examine the question of the relationship between phagocytosis and pinocytosis, we have examined these two events simultaneously and compared the impairment of these functions in the presence of inhibitors. It was discovered that uptake of particles by the ameba inhibits uptake of soluble molecules by pinocytosis, but both events are inhibited to the same extent by inhibitors of aerobic metabolism (Azide, cyanide, and 2,4 dinitrophenol) and not at all by inhibitors of glycolysis (iodoacetate and flouride). Examination of the effects of an inhibitor of protein synthesis on these two events is in progress.

3. In collaboration with Robert J. Anstead at the Goddard Space Flight Center, Greenbelt, Md., we have been examining events of capture and ingestion of particles by scanning electron microscopy. This instrument allows observation of intact, frozen-dried amebas at a resolution of approximately 500 A. The imaging system has a large depth of focus and the resulting image is a three-dimensional representation of the ameba. With stereo pairs, in particular, this kind of observation allows excellent visualization of the capture events in phagocytosis as well as the relationship of the locomotory ameba to the substrate and a much clearer indication of surface topography in the ameba.

Significance to Bio-Medical Research: Phagocytosis is a major mechanism of defense against infection and pinocytosis may possibly function in the immune response. These processes can be profitably studied in the ameba where experimental conditions are simplified, and where phagocytosis is much exaggerated.

Proposed Course of Project: We will pursue the studies on pinocytosis and begin to look more closely at surface membrane structure with the freeze-etch technique.

Publications:

Cabib, E. and Bowers, B.: Chitin and Yeast Budding. Localization of Chitin in Yeast Bud Scars. J. Biol. Chem. 246:152-159, 1971.

Serial No. NHLI-158
1. Laboratory of Biochemistry
2. Cellular Biochemistry and Ultrastructure
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Study of Motility in Ameba

Previous Serial No.: NHLI 224

Principal Investigators: Edward D. Korn
Robert R. Weihing
Thomas D. Pollard

Other Investigators: Robert S. Adelstein
W. M. Kuehl
Thomas Stossel

Cooperating Units: NHLI, Laboratory of Biochemistry, Section on Cellular Physiology
NHLI, Molecular Disease Branch

Project Description: To study the biochemical and morphological basis of movement in ameba and other motile cells.

Methods Employed: Standard methods of peptide chemistry including cyanogen bromide cleavage, gel and ion exchange chromatography, and amino acid analysis have been used to identify peptides of ameba actin. Possible effects of a new drug, cytochalasin B, on contractility have been assayed by viscometry and enzymatic analysis of purified contractile proteins and by searching for an effect on phagocytosis of polystyrene latex beads by the amebas.

Standard methods of protein chemistry have been used to identify and partially purify a myosin-like ATPase in extracts of Acanthamoeba. Its enzymatic properties are being studied using standard techniques of enzymology and its physical and chemical properties are now being investigated using gel electrophoresis, analytical ultracentrifugation and amino acid analysis. The interaction of the ameba myosin with muscle actin has been studied by enzymatic methods and preparative ultracentrifugation.

Negative staining electron microscopy has been used to study complexes of heavy meromyosin with thin filaments of Amoeba proteus and human platelets and to study the morphology of thick filaments formed by a myosin like enzyme from platelets. Phagocytosis in macrophages has been studied by the usual techniques of thin sectioning.

Major Findings:
1. Ameba actin, as described last year, resembles muscle actin in most of its

properties. It has been further characterized by the techniques of peptide chemistry. The ameba actin has been cleaved with cyanogen bromide, and the peptides separated by gel filtration. The peptide containing the unusual amino acid 3-methylhistidine has been purified by ion exchange chromatography. Its amino acid composition is very close, if not identical, to the peptide from muscle actin, designated CB-10. The peptide from ameba actin which contains the unusual amino acid ϵ-N-dimethyllysine has been partially purified. Its composition resembles that of another cyanogen bromide peptide of muscle actin designated CB-16 which does not contain ϵ-N-dimethyllysine. A third peptide of ameba actin, having a composition resembling that of CB-17 from muscle actin has also been identified.

2. Cytochalasin B is a complex mold metabolite which has been reported to inhibit movement and cytokinesis in various organisms. This inhibition is associated with the disorganization or disappearance of thin filaments thought to be associated with these movements. We have therefore investigated the effect of cytochalasin B on muscle actin. When muscle actin is polymerized in the presence of cytochalasin, the viscosity of the solution increases more rapidly than in controls, but the final viscosity reached is less than that of the control. If cytochalasin is added to a solution of actin polymer, the viscosity is lowered to a level about equal to that reached in the experiment just described. The decreases in final viscosity suggest that actin, in the presence of cytochalasin, cannot polymerize as completely as control actin. The apparent increase in the rate of polymerization induced by the presence of cytochalasin is not understood.

We have investigated the effect of cytochalasin and other compounds on phagocytosis of polystryene latex beads by the ameba. Since the ameba actively engulfs the bead during phagocytosis, one might hope to observe an effect of this inhibitor of motility on bead uptake. When phagocytosis was assayed with cells suspended in growth medium, no effect of the drug or of the dimethylsulfoxide used to dissolve it were observed.

3. Amoeba myosin: A myosin-like enzyme was identified in extracts of Acanthamoeba by fractionating a high speed centrifugal supernatant by gel filtration and then testing the several fractions which contained ATPase activity for their ability to interact with muscle F-actin in two ways: 1) ATP-reversed binding of the ATPase to F-actin as analyzed by ultracentrifugation and 2) activation of the Mg-ATPase activity of the enzyme at low ionic strength by muscle F-actin. One ATPase was found which fulfilled these criteria for myosin and it has been purified over 200 fold using ammonium sulfate fractionation and chromatography on DEAE-cellulose, Sephadex G-200 and hydroxylapatite. The enzymatic properties of the purified protein resemble those of muscle myosin: ATPase activity is highest (about 3 μmoles/min/mg protein) in 0.5M KCl in the presence of EDTA, this EDTA-ATPase activity is inhibited by lowering the KCl concentration or adding NaCl, the Ca-ATPase activity is 15% of the EDTA-ATPase activity, and 0.1mM Mg inhibits the enzyme over 98%. Like muscle myosin, the Mg-ATPase of ameba myosin is activated by muscle F-actin. This activation is dependent on the actin concentration;

it is possible to construct liner reciprocal plots of 1/(actin) vs. 1/ATPase and thereby determine the maximal actin-Mg-ATPase activity by extrapolation to infinite actin. Actin activation of over 30-fold has been measured in purified preparations. This actin activation is inhibited at higher ionic strengths as with muscle myosin. The interaction of the ameba myosin with muscle actin has also been studied by measuring binding to muscle actin in the ultracentrifuge. The enzyme appears to bind weakly to actin in 0.5M KCl, requiring high concentrations of actin to sediment in all of the ATPase. The molecular weight of the ameba myosin appears to be very different from that of any other myosin studied (including that of various muscles and slime mold MW=450,000) and has been estimated to be between 125,000 and 140,000 by Sephadex and Sepharose gel filtration in 0.5M KCl. In addition, the major band (accounting for about 70% of the protein) in SDS polyacrylamide gel electrophoresis of the most highly purified preparation corresponds to a molecular weight of 140,000. Because this molecular weight is so much smaller than that of other myosins and because muscle myosin is subject to proteolytic digestion into enzymatically active fragments, it was important to establish that the ameba myosin we have purified is not simply a fragment of a larger native myosin. Two experiments suggest, but do not prove that the native ameba myosin has a molecular weight of 140,000: a) incubation of the crude ameba homogenate for long times before centrifugation at 100,000g did not alter the yield of the EDTA/Ca-ATPase or change its partition coefficient on Sepharose 6B (a measure of molecular size); and b) the ameba homogenate does not inactivate added muscle myosin or alter its size.

4. Identification of actin in a second type of ameba: Amoeba proteus has frequently been used in studies of ameboid movement, but has not been useful for biochemical studies because of difficulties in growing mass cultures. We have indirectly identified actin in extracts of this ameba by observing complexes of heavy meromyosin with the Amoeba proteus thin filaments in the electron microscope. The repeating arrowhead complexes formed along the thin filaments are dissociated by Mg-ATP and are indistinguishable morphologically from the complex of heavy meromyosin with purified Acanthamoeba actin and muscle actin, making the tentative identification of these thin filaments as actin seem reasonable.

5. Electron microscopy of platelet myosin: In collaboration with Drs. Adelstein and Kuehl of the Section on Cellular Physiology, NHLI, myosin isolated from human platelets has been studied in the electron microscope. A high molecular weight fraction of platelet myosin forms bipolar thick filamentous aggregates at low ionic strength which are similar to aggregates of muscle myosin, having a bare central region and tufted tapered ends. A low molecular weight fraction does not form these thick filaments, but like low molecular weight fragments of muscle myosin (heavy meromyosin of subfragment) it forms characteristic arrowhead shaped complexes with muscle F-actin.

6. Electron microscopy of phagocytosis in leucocytes and macrophages: In collaboration with Drs. Stossel, Mason and Vaughan, Molecular Diseases Branch, NHLI, the phagocytosis of parafin oil emulsion by leucocytes and macrophages

has been studied. These observations showed that the emulsion is taken up into membrane bounded phagocytic vesicles into which the contents of the cells granules or lysosomes are emptied. Purified fractions of phagocytic vesicles isolated by density gradient ultracentrifugation were also examined and were found to consist of membrane bounded vesicles containing the droplets of parafin oil and granule contents. A few granules contaminated these fractions, but many of these granules were adherent to the membranes of the phagocytic vesicles. No other contamination was found.

Significance to Bio-Medical Research: An understanding of the mechanism of movement of non-muscle cells is fundamental to the understanding of such biological processes as phagocytosis, clot retraction, cell division, embryological development, cancer metastases, control of movement and localization of intracellular organelles, and mitosis.

Proposed Course of Research:
1. Ameba myosin: The purification procedure for the myosin will have to be modified to yield an essentially homogeneous protein which retains ability to be activated by actin. Purity will be evaluated by gel electrophoresis in both SDS and urea systems, electrofocussing, and analytical ultracentrifugation. The molecular weight will be determined by both SDS gel electrophoresis and equilibrium ultracentrifugation. The molecular weight behavior in gel chromatography, and viscosity will be combined with observations in the electron microscope (if possible) to evaluate the shape of the molecule. The presence of small subunits such as those found in muscle myosin will be evaluated by gel electrophoresis and comparison of the analytical ultracentrifuge behavior of the molecule in its native form and in denaturing solvents. Amino acid analysis will be done with particular attention to the possible presence of the rare methylated amino acids found in muscle myosin: 3-methylhistidine, ϵ-N-mono- and tri-methyllysines. The solubility and state of aggregation of the ATPase at low ionic strength will be studied by ultracentrifugation and electron microscopy to determine the form in which the myosin exists in the cell. More detailed enzymatic studies will be done using a pH-stat to characterize the substrate and ion specificity of the enzyme. The interaction of the ameba myosin with F-actin from both muscle and the ameba will be studied by detailed enzyme kinetic analysis, by binding studies in the ultracentrifuge and by electron microscopic observations of complexes. The influence of muscle tropomyosin on the enzymatic interaction of ameba myosin and F-actin will be determined.

Publications:

Weihing, R.R., and Korn, E.D.: ϵ-N-dimethyllysine in Amoeba Actin. Nature. 227:1263, 1970.

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Weihing, R.R., and Korn, E.D.: Acanthamoeba Actin: Isolation and Properites. Biochemistry. 10:590, 1971.

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Tech Development

Endocrinology

Chemistry

Chemical
Pharmacolc

ANNUAL REPORT OF THE
LABORATORY OF CHEMICAL PHARMACOLOGY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

DRUG METABOLISM AS A CAUSE OF DRUG TOXICITY

It is now recognized that many chemically inert organic compounds are transformed in the body to potent alkylating agents which combine with tissue components, such as proteins, lipids and nucleic acids, and thereby result in carcinogenesis and mutagenesis. This laboratory is being reorganized to explore the possibility that tissue damage caused occasionally by therapeutic and environmental substances is also mediated by reactive metabolites. To test this view, the laboratory has studied 1) whether treatments which alter the metabolism of known toxicants also alter the severity of tissue lesions, 2) whether radiolabeled toxicants become covalently bound to tissue macromolecules in vivo and 3) whether the covalently bound label is localized in the damaged areas of the tissues. We also plan to determine whether the macromolecules altered by the toxicants may serve as antigens. With these studies on the mechanism of known toxicants we hope to gain an insight into the nature of tissue lesions caused by therapeutic agents and the effects of drug interactions on such lesions.

Hepatotoxicity

Halogenated aromatic hydrocarbons: The following evidence now suggests that a number of halogenated aromatic hydrocarbons, such as bromobenzene, cause centrolobular necrosis by being converted to epoxide which in turn become covalently bound to macromolecules in liver. 1) Pretreatment of rats and mice with phenobarbital (PB), which enhances bromobenzene metabolism, increases the centrolobular necrosis caused by the toxicants, whereas simultaneous administration of β-diethylaminoethyl diphenylpropylacetate (SKF 525-A), which inhibits bromobenzene metabolism decreases the toxicity. These findings suggested that the necrosis was mediated by metabolites and not by the parent compound. 2) Autoradiographs of paraffin sections of liver after administration of ^{14}C-bromobenzene or ^{14}C-chlorobenzene showed that the label was localized in the necrotic centrolobular zone rather than in the periportal region and was in fact increased by pretreating the animals with PB and decreased by SKF 525-A. 3) Direct evidence that metabolites of ^{14}C-bromobenzene were covalently bound to liver components was obtained by exhaustive extraction of liver homogenates with methanol, isolation of radiolabeled proteins by Sephadex chromatography, enzymatic hydrolysis of the proteins and isolation of the radiolabeled amino acids. Most of the radiolabel is associated with three amino acids, which have not yet been identified.

Pretreatment of rats with 3-methylcholanthrene (3-MC) increases the amount of bromobenzene dihydrodiol and bromocatechol conjugates and decreases the amount of bromobenzene mercapturic acid excreted into urine, whereas pretreatment with PB does not alter the pattern of the urinary metabolites.

Since pretreatment of rats with 3-MC decreases the amount of covalently bound bromobenzene in liver and prevents centrolobular necrosis but does not appreciably alter the rate of bromobenzene metabolism, it seems likely that 3-MC prevents necrosis by decreasing the level of the toxic intermediate, presumably by increasing its elimination along nontoxic pathways.

Since mercapturic acids are formed from conjugates of glutathione (GSH), we studied the properties of the hepatic enzyme system which catalyze the formation of these conjugates of bromobenzene and other halogenated benzene derivatives. The results of our studies are consistent with the view that a cytochrome P-450 system in liver microsomes catalyzes the conversion of bromobenzene to its epoxide which in turn is converted to the GSH conjugate by a GSH transferase in the soluble fraction. This epoxide mediated pathway of metabolism is apparently related to liver necrosis since all those halogenated benzene derivatives which form the GSH conjugates in vitro also caused centrolobular necrosis in PB pretreated animals.

Additional biochemical evidence suggests that GSH protects animals from the toxic effects of the halogenated aromatic hydrocarbons, since most of the covalent bonding occurs only after the GSH becomes sufficiently depleted to slow the elimination of the epoxide. In accord with this view 1) the toxicity and covalent bonding of bromobenzene is markedly enhanced by prior administration of diethyl maleate which depletes liver GSH. 2) Pulse labeling experiments indicate that the pattern of metabolites changes as bromobenzene depletes the liver of GSH. 3) With toxic doses of bromobenzene there is a delay in the accumulation of covalently bound bromobenzene metabolites; the covalently bound metabolites are low during the few hours, increase exponentially and attain levels as high as 10 nmoles/mg protein at 12-24 hours.

Other hepatotoxicants: Although the formation of epoxides may mediate liver necrosis induced by aromatic hydrocarbon derivatives, they clearly cannot account for the effects of all hepatotoxicants. Nevertheless, autoradiographs revealed that covalently bound metabolites of radiolabeled CCl_4, $CHCl_3$, and thioacetamide are localized in the necrotic centrolobular regions of liver. Pretreatment with PB enhanced the binding of CCl_4 and $CHCl_3$ and their toxicity whereas SKF 525-A diminished both the binding and toxicity.

On the other hand, the periportal necrosis caused by allyl alcohol is not enhanced by PB but is prevented by prior administration of pyrazole, which presumably acts by blocking alcohol dehydrogenase. Autoradiographs of paraffin sections after the administration of radiolabeled allyl alcohol showed that large amounts of label were bound in the periportal region rather than in the centrolobular region and that pyrazole administration decreased the amount of bound radiolabel. Exhaustive extraction of liver homogenates with methanol, confirmed that the radiolabeled metabolites of allyl alcohol were covalently bound.

Effect of hepatotoxicants on liver and plasma triglycerides: In female rats liver triglycerides increase after the administration of bromobenzene, thioacetamide and allyl alcohol. Since the triglycerides were localized in the centrolobular region after administration of bromobenzene or thioacet-

amide, but in the periportal region after the administration of allyl alcohol, triglyceride accumulation occurs in the same zone as does necrosis.

Plasma triglycerides decrease after administration of thioacetamide, allyl alcohol and bromobenzene, but the decrease is delayed until 6-8 hours after the administration of allyl alcohol or bromobenzene. In accord with these findings the release of newly synthesized plasma proteins is impaired at 12 hours but not at 4 hours after bromobenzene administration.

Bone Marrow Damage

Within a few hours after the administration of the carcinogenic agent, 7,12-dimethylbenzanthracine (DMBA) to rats, DNA and protein synthesis is markedly reduced in bone marrow cells isolated from the femur, as shown by decreased incorporation of ^{3}H-thymidine and ^{14}C-labeled amino acids. Since 7,8-benzoflavone, which inhibits the metabolism of DMBA by extrahepatic tissues but not by liver, prevented the bone marrow damage, it seems likely that the toxicity is mediated by an active metabolite of DMBA. It is not yet certain whether toxic metabolite is formed directly in bone marrow itself.

Repeated injections of benzene into animals are required to cause bone marrow damage. The delay in onset of the damage may be due to an alteration in the pathways of benzene metabolism, because after a single dose, benzene is converted almost entirely to phenol whereas after repeated doses considerable amounts of benzene dihydrodiol, catechol and mercapturate are also formed. These results suggest that chronic administration induces an enzyme that leads to the formation of benzene epoxide rather than to a direct hydroxylation reaction. By contrast, pretreatment with piperonyl butoxide or PB does not appreciably affect either the rate or the pathways of benzene metabolism, but prevents the bone marrow damage. In attempting to identify the active metabolite, however, we have found that the toxicity is probably not mediated by phenol, catechol or hydroquinone; thus the mechanism of benzene-induced bone marrow damage remains unclear.

Covalent Bonding to Other Organs

Lung: Autoradiographs of paraffin sections after the administration of ^{14}C-labeled bromobenzene, chlorobenzene or naphthalene to mice showed large amounts of radiolabel bound to the bronchial epithelium. Exhaustive extraction of homogenates of lung taken from ^{14}C-bromobenzene-treated animals revealed that the amount of covalently bound metabolites in lung was about a fifth of the amount found in liver. Covalently bound bromobenzene was increased by prior administration of PB and decreased by SKF 525-A, suggesting that the alkylating agent is formed in lung tissue. In accord with this view, bromobenzene is metabolized by lung homogenates.

The carcinogenic agent DMBA also is covalently bound to lung and the amount of bound metabolites is decreased by simultaneous administration of 7,8-benzoflavone, which presumably inhibits the activation of the carcinogen in this tissue.

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Although no evidence was obtained that single doses of halogenated benzenes or DMBA cause damage to the lung, it is possible that their repeated administration may result in damage.

Kidney: Autoradiographs of paraffin sections after administration of ^{14}C-bromobenzene showed accumulation of the label in the convoluted tubules. Preliminary data suggest that bromobenzene is activated by kidney microsomes to react with GSH. These studies still underway suggest that the halogenated hydrocarbons cause kidney toxicity via an active metabolite.

Testes: Within 4 hours after administration, DMBA causes testicular necrosis, as measured by a decrease in ^{14}C-thymidine incorporation. Simultaneous administration of 7,8-benzoflavone decreases not only the necrosis but also the amount of covalently bound metabolites of DMBA.

Alterations in the Binding of Drugs to Serum Proteins

Neonatal albumin: Apparent differences in the binding of drugs by serum albumin of newborn infants and that of adults have hampered the interpretation of drug plasma levels in neonates. An investigation of sulfaphenazole binding by albumins from adults and cord blood of humans has revealed that occupancy of albumin binding sites by slowly metabolized components, such as bilirubin, in neonatal blood may account for some of the apparent differences.

Aspirin: The acetylation of serum albumin by aspirin occurs both in vitro and in vivo. Although no major changes in the conformation of the protein were discernible by optical methods and experiments with ^{14}C-aspirin showed that the binding sites for drugs were not themselves acetylated, acetylation increases the affinity of the protein for phenylbutazone and reduces that for flufenamic acid.

Effect of PB on corticosterone metabolism: Since repeated administration of PB to animals enhances the metabolism of steroids by liver microsomal enzymes, it has been suggested that PB might cause toxic reactions by decreasing the levels of glucocorticoids and other steroids. However, we have found that PB treatment of rats increases the rate of metabolism of tracer doses of corticosterone, but does not alter the plasma level of the steroid. These findings thus suggest that the mechanisms which normally control the plasma levels of corticosterone are fully able to counteract the increased corticosterone metabolism by stimulating its synthesis.

MECHANISMS OF DRUG METABOLISM

NADPH-dependent hydroxylases in liver microsomes catalyze the oxidation of a vast number of drugs and other foreign components, including many hepatotoxicants and carcinogenic agents. In the current view of the mechanism of these enzymes, the substrate combines with the oxidized form of a cytochrome, called cytochrome P-450. The complex is then reduced by NADPH by way of NADPH-cytochrome c reductase and the reduced substrate-cytochrome P-450 in turn reacts with oxygen to form an "active oxygen" complex which decomposes with the formation of the oxidized drug and the oxidized cytochrome P-450.

This mechanism implies that equivalent amounts of NADPH, oxygen and substrate are consumed in the reaction. But studies to evaluate the stoichiometric relationships have been hampered by the endogenous oxidation of NADPH. With methods devised to correct for NADPH-oxidation by enzyme systems other than cytochrome P-450, we have obtained stoichiometric relationships which approach the theoretical 1:1 relationship between the maximum oxidation of NADPH and substrate, but only when the maximum rate of substrate oxidation was estimated from Lineweaver-Burk plots. At high substrate concentrations, the oxidation of many substrates is inhibited while the substrate-stimulated NADPH-oxidation is not. The reason for the substrate inhibition is not clear but it is possible that high concentrations of substrate affect the affinity of the reduced or oxygenated forms of the cytochrome P-450 for the substrate.

Since only one electron is required to reduce the substrate-cytochrome P-450 complex, the point at which the other electron from NADPH enters the system has been puzzling. Other investigators recently suggested that the electron might enter the system after oxygenation of the reduced substrate-cytochrome P-450 complex and that the electron could come from NADH as well as NADPH. According to this view, the stoichiometric relationships in a system containing both NADH and NADPH should approach 1:1:2 for the oxidation of NADPH, NADH and substrate. However, we have found that the ratio of NADPH-oxidation to substrate oxidation was not altered by addition of NADH even though the ratio of NADH oxidation to substrate oxidation approached the theoretical 1:2 relationship. The reason for the discrepancy remains obscure.

MEMBRANES AND BIOLOGICAL CONTROL MECHANISMS

Since control of the cardiovascular system is effected through the autonomic nervous system, a goal of the laboratory is to establish the physiologic and molecular mechanisms by which signals are transfered between these systems.

Na-K-ATPase: The Na-K-ATPase from rat kidney tubules, which mediates the reabsorption of NaCl, has now been shown to form a stable complex with Mg^{++} and F^{-} ions. In this form the ATPase is inactive and remains so throughout repeated washings. Despite its inactivity, the enzyme may be converted into one of two conformations by temporary exposure to K^{+} or Na^{+}. These conformations are easily recognizable by the difference in the rate constants for the regeneration of the active state when they are exposed to 15 mM Na_2ATP, the Na^{+} dependent form being reactivated at a slower rate. Conversion of the slow form to the fast form occurs by way of an intermediate which is resistant to reactivation so long as K^{+} remains bound. Attempts to study these conformational changes of the kidney ATPase by observing the electron spin resonance spectrum of a bound, spin-labeled analogue of ATP, tetramethyl-N-oxylpiperidine-ADP, were unsuccessful but did reveal the unexpected presence of two populations of ATPase, one resistant and the other sensitive to the spin-labeled analogue.

Transport and storage of NE and 5-HT: Previous results with heart slices and isolated nerve endings have shown that transport and storage of NE and

5-HT depends on the interplay of Na^+, K^+ and Ca^{++}. The transport of the amines by the amine carrier in nerve endings requires external Na^+ and low external K^+ concentrations, but transport is blocked by high external K^+ concentrations presumably by competing with external Na^+ for the amine carrier. The recent finding in our laboratory that Na^+ rapidly diffuses across the synaptosomal wall suggested that the K^+ gradient is more important than the Na^+ gradient in the transport of the amines. In accord with this view, ouabain, which blocks the Na-K-ATPase almost instantaneously, causes depletion of internal K^+ without appreciably affecting the internal Na^+ concentrations. Moreover, in a K^+-free medium containing Na^+, transport of the amines ceases after the synaptosomes are depleted of K^+.

Storage of the amines by synaptosomes and tissues also requires Na^+, but the effects of Na^+ on storage are noncompetitively antagonized by Ca^{++}. Accordingly, in media containing Ca^{++} and low Na^+ concentrations, Ca^{++} enters synaptosomes as the amines and internal Na^+ are released. Presumably the internal Ca^{++} then causes release of the amines from the synaptic vesicles. The mechanism by which Ca^{++} is extruded from the synaptosomes, however, remains unknown.

Cyclic AMP as a mediator of target organs: Cyclic AMP is known to mediate the action of hormones and neurotransmitters in a number of tissues such as fat cells, brain, lung and liver. Last year it was reported that insulin did not change the initial rate of formation of radiolabeled cyclic AMP induced by NE, but that it increases the rate at which radiolabeled cyclic AMP disappears from intact cells of adipose tissue. These results were confirmed with the recently developed method of Gilman, which measures total amounts of cyclic AMP. However, the ratio of the specific activity of radiolabeled cyclic AMP to that of radiolabeled ATP in cells from adipose tissue was considerably less than one at low NE concentrations but the ratio increased as the NE concentrations were increased. These findings suggest that high NE concentrations mobilize pools of ATP that are not used at low NE concentrations.

β-Adrenergic receptor: Since both α- and β-recetpors are present in aortic smooth muscle, the response to a dual agonist, such as epinephrine or isoproterenol, represents the net effect of mutually antagonistic contractile and relaxing forces. Tachyphylaxis to the β-response could explain the observations, made in this laboratory, that upon repeated administration of isoproterenol relaxation diminishes and contractile responses appear. A number of drugs, including α-antagonists and agents presumed to relax muscle by several mechanisms, prevent the appearance of contractile responses to repeated treatment with isoproterenol. Preliminary dose-response data suggest, however, that prevention of tachyphylaxis by some of these organic amines occurs by a mechanism ordinarily not attributed to them. In other studies β-responses in rat aorta have been shown to diminish with age at rates which differ in various strains.

Metabolism of acetylcholine in brain: With the use of the sensitive analytical method developed last year for the assay of acetylcholine and choline, it was discovered that pilocarpine increases the brain levels of acetylcholine. The drug presumably acts by preventing the release of acetylcholine since it blocks acetylcholine synthesis and does not inhibit choline-

sterase.

Since brain levels of choline are usually less than the K_m value of choline acetyl transferase, it seems possible that the rate of acetylcholine synthesis might be limited by the uptake of choline into brain tissues. Studies on the uptake of ^{14}C-choline into slices from various parts of the brain showed that the rate of choline uptake was highest in the striatum and lowest in the cerebellum. In the striatum, choline is rapidly converted to acetylcholine; within 5 minutes about 30% of the radiolabeled choline is present as acetylcholine.

Physicochemical studies of drug-receptor interactions: These included a) the continued development of physical methods for studying the interaction of drugs with membranes and b) the use of physical methods to elucidate the interaction of drugs with purified protein "receptors" of general biological interest.

a) Fluorescent "probes" have been used to probe the effects of drugs on the hydrocarbon, glycerol and aqueous interfacial regions of the bilayered membrane of human red cells. Butacaine perturbs primarily the hydrophobic regions of the red cell membrane whereas the closely related tetracaine causes much more widespread damage. Flufenamic acid, an anti-inflammatory agent that stabilizes red cell membranes at low concentrations but labilizes them at high concentrations, did not affect fluorescence of a probe presumed to label the hydrophobic portion of the membrane but apparently displaced another probe which presumably binds in the glycerol region of the bilayer.

b) The relative strength of binding of several cholchicine analogues to microtubular portein purified from brain has been compared with the relative antimitotic and anti-inflammatory effects of these compounds. For example, those substances which readily displace colchicine from the microtubular protein were effective in preventing the edema caused by injection of sodium urate crystals into rat paws. These and other correlations indicate that the binding site on microtubules must be similar in brain, mitotic spindles and polymorphonuclear leucocytes. Occupancy of this binding site by cholchicine requires a dimeric form of this ubiquitous protein and prevents assembly into the tubular form which functions in the transport of NE storage granules along the axon toward the site of their eventual release.

A spin-labeled sulfonamide has been synthesized and found to bind in a 1:1 complex with bovine erythrocyte carbonic anhydrase by fluorescence titration procedures that have been developed in this laboratory. This binding results in noncompetitive inhibition of the enzyme. Electron spin resonance spectra indicate strong immobilization of the inhibitor, presumably in a narrow crevice which X-ray studies have shown to contain the active site. With the synthetic procedures developed in this laboratory we shall prepare spin-labeled homologues with variable distances separating the stable free radical and the sulfonamide. With these homologues it will be possible to probe the depth of the central cavity by determining that chain length which just permits increased degrees of freedom to the emergent spin label. In this way the information derived from the use of spin labels can be correlated with that obtainable from X-ray diffraction.

Serial No. NHLI-159
1. Chemical Pharmacology
2. Enzyme Drug Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An investigation of the mechanisms by which various steroids alter the hepatic microsomal mixed function oxidase system to alter drug metabolism

Previous Serial Number: NHLI-275

Principal Investigators: Dr. Maynard E. Hamrick
Dr. Nicola G. Zampaglione
Dr. Bitten Stripp
Dr. James R. Gillette

Other Investigators: None

Cooperating Unit: Drs. Hamrick and Zampaglione are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Previous studies have indicated that various hormones regulate different steps in the sequence of events by which the hepatic mixed function oxidase system metabolizes drugs. For example, testosterone is thought to alter the binding of drugs in the type I category to cytochrome P-450 as indicated by an increase in the type I spectral change. This increase in the binding of the drug to cytochrome P-450 allows a greater rate of reduction of cytochrome P-450 since the P-450-substrate complex is reduced more rapidly than P-450 alone. The increased reduction rate results in an increased rate of drug metabolism. Another group of hormones, the glucocorticoids, are thought to increase the activity of NADPH-cytochrome c reductase, which in turn increases the rate of reduction of cytochrome P-450 and allows a greater rate of drug metabolism.

In this study the effects of spironolactone, methyl-testosterone, and cortisone on the type I spectra, NADPH-cytochrome c reductase, P-450 reductase, cytochrome P-450 content, NADPH oxidation, and the metabolism of hexobarbital and ethylmorphine were investigated. The objective of this investigation was to elucidate the effects of testosterone and cortisone on the hepatic mixed function oxidase system and to compare their actions with the actions of spironolactone on this system.

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Methods Employed: Male and female Sprague-Dawley rats were used in this study. Groups of each sex were pretreated with methyl-testosterone, cortisone, or spironolactone on two different dosage schedules. For acute pretreatment of animals weighing 160 to 180 g, 100 mg/kg of the drugs were injected intraperitoneally twice per day for four days. For chronic pretreatment of animals initially weighing 50 g, 20 mg/kg of the drugs were injected subcutaneously in oil each day for four weeks. These animals were sexually mature by the end of the experiment.

To determine the stoichiometric relationship between NADPH oxidation and drug metabolism in microsomal suspensions, the maximal rates of drug metabolism in air were determined, and the rate of NADPH oxidation was measured both in the presence of V_{max} concentrations of substrates in air and in the absence of substrate in a $CO:O_2$ (9:1) atmosphere. The rates of metabolism and the P-450 dependent NADPH oxidation were then related as follows:

$$\frac{\text{(Metabolism + Air)}}{\text{(NADPH Oxid. + Substrate + Air) - (NADPH Oxid. + CO)}}$$

Standard biochemical techniques were employed.

Major Findings:

1. P-450 Content: The P-450 content was not changed to any great extent in the males or females by methyl-testosterone, spironolactone, or cortisone.

2. Stimulation of P-450 Reductase by Substrates: In control animals the stimulation of P-450 reductase by substrates was sex dependent. The P-450 reductase was stimulated approximately 30 percent in females and 100 percent in males.

Following the acute pretreatment of the female rats with any of the three steroids, the stimulation of P-450 reductase by hexobarbital and ethylmorphine was increased. When compared to controls, the methyl-testosterone and cortisone pretreatment approximately doubled the stimulability of P-450 reductase, while spironolactone tripled the stimulability. The acute pretreatment in males also increased the P-450 reductase stimulability by substrates, but only about 25 percent.

In chronically pretreated male and female rats, the cortisone did not increase the P-450 reductase stimulability, while both the methyl-testosterone and spironolactone doubled the P-450 reductase stimulability. Interestingly, the P-450 reductase stimulability of all the groups was depressed by the chronic pretreatment as compared to the values obtained after the acute pretreatment.

Both spironolactone and methyl-testosterone increased the P-450 reductase stimulability of female rats to a value approximately equal to the P-450 reductase stimulability observed in male rats whether the substrate

was hexobarbital or ethylmorphine. However, only ethylmorphine metabolism was increased to the level observed in males.

3. NADPH-Cytochrome c Reductase: Following acute pretreatment of male and female rats cortisone and spironolactone increased the NADPH-cytochrome c reductase activity by 50 to 100 percent. Methyl-testosterone did not significantly increase this reductase activity. After chronic pretreatment of the male and female rats, the NADPH-cytochrome c reductase activity was not increased by any of the three pretreatments.

4. Stoichiometric Relationship Between NADPH Oxidation and Drug Metabolism: In male controls the stoichiometric relationship between drug metabolism and NADPH oxidation was approximately 1:1 for both hexobarbital and ethylmorphine. In females the relationship was approximately 0.50:1.0 for ethylmorphine.

Methyl-testosterone or spironolactone pretreatment of females increased the ratio for both hexobarbital and ethylmorphine toward 1:1, but methyl-testosterone was more effective especially in chronically pretreated females. Cortisone did not alter the ratio in females.

The stoichiometric relationship between drug metabolism and NADPH oxidation in males was decreased following spironolactone pretreatment for both hexobarbital and ethylmorphine to a ratio similar to those found in the female control animals. Cortisone pretreatment also decreased the ratio for both substrates but to a lesser extent than did spironolactone. Methyl-testosterone maintained the ratio at approximately 1:1. So, the spironolactone and cortisone apparently act to block some action of methyl-testosterone on the mixed function oxidase system in male rats.

5. Binding of Type I Substrates to Cytochrome P-450: A sex difference in the A_{max} of both hexobarbital and ethylmorphine to cytochrome P-450 was observed as indicated by the change in the type I spectra. Spironolactone pretreatment of female rats doubled the binding of hexobarbital to cytochrome P-450 to a value approximately the same as in males but only slightly increased the binding of ethylmorphine to cytochrome P-450. Methyl-testosterone also increased the binding of both substrates, especially following the chronic pretreatment. Cortisone had no effect on the binding in females following the acute pretreatment but did double the binding of hexobarbital after chronic pretreatment.

In male rats spironolactone did not alter the A_{max} of either substrate. Methyl-testosterone produced a 10 to 20 percent increase in the binding of both substrates in males. Cortisone decreased the binding of both substrates in males. So, spironolactone and methyl-testosterone apparently act in a similar manner to increase binding of substrate to P-450, while cortisone apparently competes with methyl-testosterone in males to decrease the binding of substrate to cytochrome P-450.

Significance to Biomedical Research and the Program of the Institute: This study should provide a better understanding of the mechanisms by which hormones alter drug metabolism.

Proposed Course of Project: This project has been terminated.

Honors and Awards: Dr. Gillette will be a Claude Bernard Professor, University of Montreal, April 22 and 23, 1971.

Publications:

Stripp, B., Hamrick, M., Zampaglione, N., and Gillette, J.R.: The effect of spironolactone on drug metabolism by hepatic microsomes. J. Pharmacol. Exp. Ther. 176: 766-771, 1971.

Serial No. NHLI-160
1. Chemical Pharmacology
2. Enzyme Drug Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the oxidation of NADH stimulated by NADPH and drugs in liver microsomes

Previous Serial Number: None

Principal Investigators: Dr. Henry Sasame
Dr. James R. Gillette

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Hepatic microsomal cytochrome P-450 has been implicated as the terminal oxidase in the metabolism of steroids, drugs and other foreign compounds. In the current view of the mechanism of this enzyme, equivalent amounts of NADPH, substrate and oxygen are consumed in the reaction. Since the reduction of cytochrome P-450 requires only one electron, it seems likely that the other electron reduces another site of the enzyme system following the reduction of P-450. Recently, Hildebrandt et al. (Fed. Proc. 29: 138, 1970) have suggested that this other site may be reduced preferentially by NADH as well as by NADPH, since the mixture containing both NADPH and drug substrate greatly enhanced the rate of NADH oxidation.

Furthermore, the finding that drug substrates in the presence of NADPH decreased the steady-state levels of reduced cytochrome b_5 in liver microsomes and that NADH increased it led Dr. Estabrook to propose that the extra electron from NADH may be mediated by cytochrome b_5. According to this mechanism, however, the stoichiometric relationship for the oxidation of NADPH, NADH and substrate should approach 1:1:2. The purpose of this project was to determine the validity of this mechanism.

Methods Employed: The stoichiometric relationship between the formation of formaldehyde and oxidations of NADPH and NADH involved during the course of N-demethylation of either ethylmorphine or aminopyrine by rat liver microsomes was investigated in air and in an atmosphere consisting of 90% CO and 10% oxygen. In order to eliminate a quenching effect of microsomal protein on the spectrophotometer determination of pyridine nucleotides at 340 mμ, the incubation mixtures were assayed for the formation of formaldehyde by

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by the Nash method, for NAD by alcohol dehydrogenase method and for NADP by glucose-6-phosphate dehydrogenase method. The amounts of NADPH, NADH and substrate oxidized in the presence of CO were then subtracted from the values obtained in air.

Major Findings: Although the steady-state level of cytochrome b_5 in liver microsomes was lowered considerably under the combined effect of NADH, NADPH and a substrate, there was no decrease in the steady-state level of cytochrome P-450-CO complex, which was in an equilibrium with cytochrome $P\text{-}450_{red}\text{-}O_2$-substrate complex. In accordance with this observation, NADH did not increase the rate of NADPH-dependent cytochrome P-450 reduction, which is presumably a rate-limiting step, or stimulate the rate of reoxidation of reduced P-450-CO complex.

In the absence of NADH, the ratio of the NADPH oxidation to aminopyrine demethylation was about 1.4 but the addition of NADH did not decrease this ratio even though the ratio of NADH oxidation to substrate oxidation approached the theoretical ratio of 1:2. Similar results were also obtained with ethylmorphine as a substrate. These findings strongly suggest that the drug deviates a flow of electron from NADPH into some yet unidentified pathway, which is also carbon-monoxide sensitive.

Significance to Biomedical Research and the Program of the Institute: An understanding of the mechanism of the cytochrome P-450 enzymes in liver microsomes may be important in the understanding of individual variations in drug metabolism.

Proposed Course of Project: The project has been terminated.

Honors and Awards: None

Publications: Sasame, H.A. and Gillette, J.R.: Studies on the inhibitory effects of various substances on drug metabolism by liver microsomes: The effect of nicotinamide in altering the apparent mechanism of inhibition. Biochem. Pharmacol. 19: 1025-1041, 1970

Sasame, H.A. and Gillette, J.R.: The inhibitory effects of endogenous NADPH-pyrophosphatase on the reduction of cytochrome c and the oxidation of drugs by liver microsomes. Arch. Biochem. Biophys. 140: 113-121, 1971.

Serial No. NHLI-161
1. Chemical Pharmacology
2. Enzyme Drug Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An approach to measure the stoichiometric relationship between hepatic microsomal drug metabolism and NADPH oxidation

Previous Serial Number: None

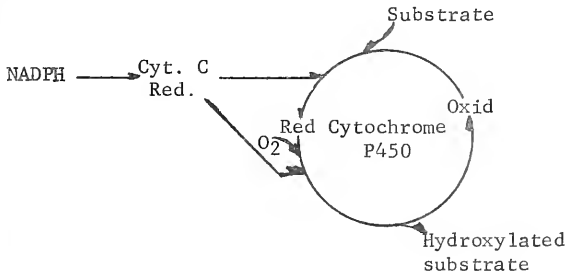
Principal Investigators: Dr. Bitten Stripp
Dr. Maynard E. Hamrick
Dr. Nicola G. Zampaglione
Dr. James R. Gillette

Other Investigators: None

Cooperating Unit: Drs. Hamrick and Zampaglione are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: The NADPH dependent hepatic microsome enzymes responsible for the metabolism of a large number of drugs are often referred to as mixed function oxidases (MFO). According to the definition of MFO by Mason, this implies a 1:1 relationship between the amounts of drug metabolized and NADPH oxidized. This stoichiometry has been shown for only a few compounds and in a recent study with spironolactone (NHLI-275), we found a close 1:1 relationship with ethylmorphine but not with hexobarbital. Both of these are so-called type I compounds and are assumed to be metabolized according to fig. 1. The



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endogenous rate of NADPH oxidation may or may not be involved in metabolism of substrate and therefore a true relationship between metabolites formed and the related NADPH oxidation cannot be obtained simply by measuring substrate-stimulated NADPH oxidation.

The purpose of this project was to develop a relatively quick and simple method to estimate the NADPH utilization related to the oxidative metabolism in the P-450 dependent electron transport system.

Methods Employed: Standard biochemical techniques were employed. The V_{max} was measured in air and in an atmosphere of $O_2/CO = 1/9$. The NADPH oxidation was measured endogenously and in the presence of maximal stimulating concentrations of substrates, and both of these measurements were also carried out in the O_2/CO atmosphere The inhibitory effect of CO both on the endogenous and on the substrate-stimulated NADPH oxidation was utilized to derive two formulas, one of which related the metabolism to a corrected substrate dependent NADPH oxidation; the other related the CO sensitive metabolism to the CO sensitive, substrate dependent NADPH oxidation.

First formula: Let A represent the endogenous NADPH oxidation and be the sum of the oxidation through three different pathways:

$$A = "S" + N + U$$

Where "S" is the part that can be stimulated by substrate and become directly related to metabolism, N is the part not to be stimulated by substrate but related to P-450 and U represents pathways that are unrelated to substrate and P-450. The "S" pathway can be subdivided into a CO-inhibitable part S and a CO-resistant part XS. Thus, in presence of substrate, let the NADPH oxidation be $B = S^* + XS^* + N + U$. In presence of O_2/CO, let the rate equal $C = XS + U$ and finally in the presence of O_2/CO and substrate, let the rate of oxidation equal $D = XS^* + U$. By subtracting C from B, one gets $C - B = S^* + XS^* + N - XS$; the true substrate related NADPH oxidation is by definition $S^* + XS^*$. Thus, error of B - C as a measure of V_{max} will be N - XS. N - XS, however, are of opposite signs and furthermore are assumed to be small compared to $S^* + XS^*$; thus, the first formula is

$$\frac{V_{max_{air}}}{[\text{NADPH OXID. + SUBSTRATE IN AIR}] - [\text{NADPH OXID. + } O_2/CO]}$$

By the same reasoning, one arrives to the second formula, which reads: $\frac{V_{max_{air}} - V_{max_{O_2/CO}}}{B - D}$. Here $B - D = S^* + N$; S^* is by definition the CO-inhibitable metabolism. Thus, by dividing $S^* + N$, the denominator will be overestimated by N.

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Major Findings: The two formulas were used to evaluate the stoichiometry of the metabolism of ethylmorphine, aminopyrine, benzphetamine, p-chloro-N-methylaniline, imipramine and hexobarbital. Values close to 1 were obtained for all substrates with both formulas. In most cases, the exact value of 1.00 was not obtained, not only because of the already mentioned small errors in the formula, but also because the metabolism of the substrates (except for imipramine and hexobarbital for which the disappearance methods were used) was measured by their N-demethylation and not by their total metabolism. The importance of using V_{max} rather than a V at saturated substrate concentration becomes evident for substrates like p-chloro-N-methylaniline and hexobarbital where the velocity never gets close to the calculated V_{max}, either because of substrate inhibition or protein precipitation at high substrate concentrations. These high substrate concentrations, however, do not inhibit NADPH oxidation, and this could be the reason why in previous studies hexobarbital was found to have a different relationship to NADPH oxidation than ethylmorphine.

Significance to Biomedical Research and the Program of the Institute: The two formulas provide a tool for measuring the effectiveness of the NADPH dependent hepatic microsomal enzyme system. In another study, it is shown that different hormonal treatments of rats seem to alter their effectiveness.

Proposed Course of Project: Has been terminated.

Honors and Awards: None

Publications:

Castro, J.A., Greene, F.E., Gigon, P., Sasame, H. and Gillette, J.R.: Effect of adrenalectomy and cortisone administration on components of the liver microsomal mixed function oxygenase system of male rats which catalyzes ethylmorphine metabolism. Biochem. Pharmacol. 19: 2461-2467, 1970.

Serial No. NHLI-162
1. Chemical Pharmacology
2. Enzyme Drug Interaction
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	A comparison of the effects of halothane and CCl_4 on the hepatic drug metabolizing system
Previous Serial Number:	NHLI-269
Principal Investigators:	Dr. Donald C. Davis Dr. David H. Schroeder Dr. Theodore E. Gram Dr. James R. Gillette
Other Investigators:	Major Reginald L. Reagan
Cooperating Units:	Drs. Davis and Schroeder are Research Associates in the Pharmacology-Toxicology Program, NIGMS Major Reagan is with the USPHS, NCI

Project Description:

Objectives: An investigation was undertaken to compare the effects of halothane and CCl_4 on various components of the drug metabolism system in hepatic microsomes and to study the influence of phenobarbital pretreatment on these effects.

Methods Employed: Ethylmorphine metabolism was measured by determining the amount of formaldehyde liberated; aniline metabolism was assessed by measuring the formation of p-aminophenol. Cytochrome P-450 content and NADPH cytochrome c reductase activity were measured employing spectroscopic methods. Electron micrographs were prepared using an RCA EMU-3G electron microscope.

Major Findings: Both halothane and CCl_4 significantly decreased microsomal ethylmorphine demethylase, NADPH-cytochrome c reductase, and cytochrome P-450, but the effects were considerably less pronounced with halothane. After phenobarbital pretreatment, halothane had no significant effect on microsomal enzyme activities, but the effects of CCl_4 were enhanced. Thus, although impairment of microsomal drug metabolism by CCl_4 appears to be mediated by an active metabolite whose formation is stimulated by phenobarbital pretreatment, halothane may act through a different mechanism.

Significance to Biomedical Research and the Program of the Institute: Little information is available concerning the toxicity of halothane at a molecular level. Since halothane is often administered clinically as an

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anesthetic to patients who are receiving a variety of drugs, it is important to understand the effects of halothane on the metabolism of other drugs in order to assess possible drug toxicities and interactions.

Proposed Course of Project: This project per se has been terminated but studies on the interaction of other anesthetic agents with the drug metabolizing system will continue.

Honors and Awards: None

Publications: Davis, D.C., Schroeder, D.H., Gram, T.E., Reagan, R.L., and Gillette, J.R.: A comparison of the effects of halothane and CCl_4 on the hepatic drug metabolizing system. J. Pharmacol. Exp. Ther., in press.

Gram, T.E., Guarino, A.M., Schroeder, D.H., Davis, D.C., Reagan, R.L. and Gillette, J.R.: The effect of starvation on the kinetics of drug oxidation by hepatic microsomal enzymes from male and female rats. J. Pharmacol. Exp. Therap. 175: 12-21, 1970.

Guarino, A.M., Schroeder, D.H., Adamson, R.H., Call, J.B. and Gram, T.E.: Studies of hepatic microsomal enzymes, serum proteins, and serum cholesterol after treatment of rats with l-asparaginase alone or in combination with phenobarbital. J. Nat. Cancer Inst. 45: 783-787, 1970.

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Serial No. NHLI-163
1. Chemical Pharmacology
2. Enzyme Drug Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Resolution of Components of Mixed Function Oxidases Concerned with Drug Metabolism

Previous Serial Number: NHLI-274

Principal Investigators: Dr. David Jollow
Dr. James R. Gillette

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Last year we reported that studies on the purification of the mixed function oxidases concerned with drug metabolism were hampered by activation of the enzymes during the purification process. Activation appeared to be dependent on the physical state of the submembrane particles and varied appreciably from preparation to preparation, obscuring the degree of purification. Similar activation of the drug metabolizing system could be demonstrated in intact microsomal membranes by including high concentrations of salts in the reaction mixture.

The present studies were designed to investigate the nature of the activation phenomenon.

Methods Employed: Standard biochemical techniques were employed.

Major Findings: 1. Increase in the concentration of potassium phosphate, sodium sulphate or potassium chloride in the reaction medium causes a marked stimulation (Ca 220%) of aminopyrine and ethylmorphine N-demethylase activities of rat liver microsomes. Activation is independent of the presence of Versene (5×10^{-3}M) and appears to be related only to the ionic strength of the reaction medium. 2. The effect of increasing ionic strength has been studied on the following parameters: kinetic analysis of drug demethylation, spectral titration, cytochrome-c reductase activity, endogenous and substrate-stimulated NADPH oxidase activities, and endogenous and substrate-stimulated cytochrome P-450 reductase activities. Comparison of these various parameters indicate that the activation phenomenon is complex and is due to the combination of several effects. 3. Comparison of kinetic analysis of drug metabolism with spectral affinity titrations suggests that about 15% of the increased metabolism may be due to an increased availability of substrate binding sites on the microsomal membrane. 4. Comparison of the

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various enzyme activities and their stimulation by increasing ionic strength in the reaction medium indicates that part of the activation phenomenon is due to a marked stimulation of cytochrome P-450 reductase activity, relieving the rate-limiting nature of this enzymic step in the overall drug-metabolizing pathway. 5. Further increase in the ionic strength of the reaction medium appears to suppress the endogenous NADPH oxidase activity resulting in a facilitated flow of reducing equivalents to the exogenous (drug) substrate.

Significance to Biomedical Research and the Program of the Institu e: Knowledge of the nature of the enzymes concerned with drug metabolism should provide a more rational basis for drug design and for assessing the various environmental influences on drug metabolism.

Proposed Course of Project: This project will be terminated.

Honors and Awards: None

Publications: None

Serial No. NHLI-164
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Autoradiographic Studies on the Localization of ^{14}C-Labeled Hepatotoxins

Previous Serial Number: None

Principal Investigator: Dr. Watson D. Reid

Other Investigators: Mr. John George

Cooperating Units: None

Project Description:

Objectives: Recent studies in this laboratory indicate that bromobenzene and other hepatotoxins are metabolized to more toxic intermediates capable of reacting covalently with macromolecules in the liver. Autoradiography was used as a tool to determine the covalently bound radioactive material was localized in the areas of tissue damage.

Methods Employed: C-57 black/6 mice (males, 20 g) or Sprague-Dawley rats (males, 180 g) were killed 24 hr after i.p. administration of 20-100 uCi of various compounds in doses previously shown to produce hepatic necrosis. Slices of liver and of various other organs were fixed in buffered formalin. Paraffin sections 6-8 microns in thickness were mounted on glass slides, deparaffinized, dipped in Kodak NTB emulsion, exposed for 2-8 weeks, developed and stained with hematoxylin and eosin. Since preparation of the sections involves repeated washing in organic solvents it is unlikely that significant amounts of unbound radioactive material remained in the tissue sections.

Major Findings: ^{14}C-Labeled metabolites of bromobenzene, chlorobenzene, naphthalene, carbon tetrachloride and chloroform - all compounds which produce centrolobular necrosis were localized almost entirely in the zone of hepatocytes surrounding the centrolobular vein. The binding appeared to be quantitatively greater and more widespread throughout the lobule when mice were pretreated with phenobarbital (80 mg/kg, i.p. for 3 days), which induces cytochrome P-450 microsomal enzymes. In contrast, pretreatment with SKF 525-A (75 mg/kg, i.p. every 8 hr), a compound which inhibits microsomal enzymes, markedly

reduced the amount of ^{14}C-bound to tissues.

The ^{14}C-aromatic compounds were also localized in the convoluted tubules of the kidney and in the bronchial epithelium of lung. No significant labeling occurred in any other tissues (brain, spleen, stomach, small intestine, adrenals, heart).

Significance to Biomedical Research and to the Program of the Institute: These studies suggest that the covalent binding of a compound to cellular constituents is in some way related to the hepatotoxic effects of the compounds. It is possible that alkylation of tissue macromolecules is an important mechanism in the production of tissue damage by a wide variety of chemicals, including therapeutic drugs.

Proposed Course of Project: Autoradiography will continue to be an important tool to compliment chemical studies on the covalent binding of foreign compounds, or their metabolites to tissues. We plan to study the toxic mechanisms of a wide variety of industrial solvents, carcinogens, environmental pollutants, food additives and therapeutic agents.

Honors and Awards: None

Publications: Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G. and Gillette, J.R.: Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc. Nat. Acad. Sci. 68: 160-164, 1971.

Serial No. NHLI-165
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of 3-Methylcholanthrene Hepatotoxic Effects Caused by Various Toxicants in Rats and Mice

Previous Serial Number: None

Principal Investigators: Dr. Watson D. Reid
Dr. Gopal Krishna
Dr. Basil Christie
Dr. Fritz Michel Eichelbaum
Mr. John George

Other Investigators: None

Cooperating Units: Dr. Eichelbaum holds a Geigy Fellowship

Project Description:

Objectives: Bromobenzene-induced hepatic centrolobular necrosis is potentiated by pretreating rats or mice with phenobarbital, an agent known to induce a wide variety of microsomal enzymes in the liver. Studies in this laboratory have shown that necrosis is probably caused by a chemically active metabolite of bromobenzene which reacts covalently with sulfhydral groups of liver proteins. 3-Methylcholanthrene (3-MC) also induces certain microsomal enzymes and it was expected that this compound would potentiate the hepatotoxicity of various liver toxins. However, we found that 3-MC administration prevented the hepatotoxicity of bromobenzene and further studies were undertaken to investigate the mechanism of this effect.

Methods Employed: Three doses of 3-MC 920 mg/kg, i.p.) were administered 88, 72 and 64 hr before the i.p. injection of ^{14}C-bromobenzene in doses known to produce necrosis in rats and mice. Livers were removed, sections taken for histology and the covalent binding of radioactive material determined as described in the NHLI-245 report (1970). The level of unbound bromobenzene was determined in plasma and liver, and the amount of total urinary metabolites and of p-bromophenylmercapturic was measured as describe in previous reports.

Major Findings: Prior administration of 3-MC to rats completely blocked the necrosis elicited by bromobenzene, chlorobenzene, and α-chloronaphthalene, reduced slightly the severity of carbon tetrachloride-induced necrotic lesions but had no effect on the hepatotoxicity of dimethylnitrosamine or thioacetamide. In contrast 3-MC had no effect in mice on bromobenzene toxicity.

Binding studies in vivo indicated that 3-MC reduced the covalently bound material in the liver of rats by 50% but had no effect on, or increased slightly , the binding in mouse liver. The disappearance of ^{14}C-bromobenzene from plasma and liver of rats and the 24 hr excretion of total metabolites was enhanced by 3-MC administration. However, the excretion of p-bromophenylmercapturic acid fell by about one third.

Significance to Biomedical Research and the Program of the Institute: These results suggest that 3-MC administration increases the overall rate of bromobenzene metabolism but reduces metabolic pathway leading to tissue damage, since both the binding to tissue and the alkylation of glutathione by bromobenzene epoxide to form a mercapturic acid are decreased. These results indicate that 3-MC may be an important tool in understanding the mechanism of bromobenzene-induced necrosis.

Proposed Course of Project: The possibility that treatment with 3-MC reduces the binding of bromobenzene epoxide to tissue macromolecules by enhancing the conversion of the epoxide to nontoxic derivatives such as the dihydrodial, catechol, and phenol is being investigated.

Honors and Awards: None

Publications: None.

Serial No. NHLI-166
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Relationship between Bromobenzene Metabolism and Hepatic Necrosis

Previous Serial Number: NHLI-245

Principal Investigators: Dr. Watson D. Reid
Dr. Jerry M. Mitchell
Dr. Basil Christie
Dr. Gopal Krishna

Other Investigators: Dr. Jay Moskowitz
Mr. John George

Cooperating Units: Drs. Jerry M. Mitchell and Jay Moskowitz are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Recent studies in this laboratory suggest that hepatic microsomal enzymes metabolize bromobenzene and other inert halogenated aromatic hydrocarbons to chemically active intermediates which produce centrolobular hepatic necrosis in vivo and which react covalently with glutathione in vitro. The present experiments lend further support to this hypothesis by demonstrating that the severity of bromobenzene-induced hepatic necrosis depends on the rate of metabolism of the methods.

Male Sprague-Dawley rats (160-200 g) were killed at various times after the i.p. administration of bromobenzene in sesame oil. In some experiments the hydrocarbon was labeled with either ^{3}H or ^{14}C. Paraffin sections of the liver were treated with periodic acid - Schiff reagent to stain glycogen in cytoplasm and with hematoxylin to counterstain nucleic. Bromobenzene concentration in a heptane extract of plasma or tissue homogenates was estimated either by gas-liquid chromatography using an electron capture detector, or from the specific activity of labeled bromobenzene. Urinary metabolites of ^{14}C-bromobenzene were isolated by extraction and chromatographic techniques.

Major Findings: Induction of hepatic microsomal enzymes by prior administration of phenobarbital (80 mg/kg, i.e. for 3 days) markedly en-

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hanced the rate of bromobenzene disappearance from plasma, liver and fat, whereas treatment with either SKF 525-A (75 mg/kg) or piperonyl butoxide (340 mg/kg) every 8 hr during the experiment prolonged the half-life of bromobenzene in plasma and tissues.

After phenobarbital administration the excretion of total metabolites and p-bromophenylmercapturic acid of ^{14}C-bromobenzene was increased 5-fold during the first 4 hr and 3-fold during the next 4 hr after injection of the hydrocarbon, although the total metabolism was the same in induced and control groups after 24 hr. Administration of SKF 525-A or of piperonyl butoxide reduced the excretion of ^{14}C-bromobenzene metabolites by one-third to one-half.

Induction of microsomal enzymes dramatically increased the hepatotoxicity of bromobenzene. Not only was the extent and severity of the centrolobular necrosis much greater in the induc-d animals, but the necrotic changes developed within 8 hr compared with 24 hr in control animals. In contrast, both SKF 525-A and piperonyl butoxide completely prevented the necrosis produced by bromobenzene (75 mg/kg).

Significance to Biomedical Research and to the Program of the Institute: These results indicate that the hepatic necrosis following bromobenzene administration is produced by a metabolite and not by the parent compound. The finding that the rate of p-bromophenylmercapturic acid excretion is increased by phenobarbital probably reflects an enhanced rate of formation of bromobenzene epoxide, a metabolite which reacts covalently with glutathione to form the mercapturate. We postulate that when microsomal enzymes are induced the rate of epoxide formation exceeds the availability of glutathione with the result that more epoxide is free to alkylate sulfhydral groups in tissue macromolecules.

Proposed Course of Project: Further studies are being carried out to gain a broader insight into the possible causal relationship between the covalent binding of metabolites of various hepatotoxins to tissue macromolecules and the tissue lesions caused by these compounds. We plan to use a similar approach in studying the tissue lesions caused by therapeutic agents.

Honors and Awards: None

Publication: Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G. and Gillette, J.R.: Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc. Nat. Acad. Sci. 68: 160-164, 1971.

Serial No. NHLI-167
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Covalent Binding of ^{14}C-bromobenzene in vivo

Previous Serial Number: None

Principal Investigators: Dr. Watson D. Reid
Mr. John George

Other Investigators: Dr. Gopal Krishna
Dr. Fritz Michel Eichelbaum

Cooperating Unit: Dr. F.M. Eichelbaum holds a Geigy Fellowship

Project Description:

Objectives: We have postulated that bromobenzene produces centrolobular necrosis through the covalent binding of a chemically reactive metabolite to tissue macromolecules. The present studies provide direct evidence of a strong bond between a metabolite of ^{14}C-bromobenzene and macromolecules in the liver.

Methods Employed: Male C-57 Black/6 mice weighing 20 g were injected, i.p. with ^{14}C-bromobenzene dissolved in 0.2 ml of sesame oil. The animals were killed at various times and a section of the liver taken for histology. The remainder of the liver was homogenized in 4 volumes of saline and the homogenate extracted 3 times with 5 volumes of heptane. Proteins were precipitated with 10% TCA and the pellet was suspended in methanol at 60° and shaken for 10 min. After 5 extractions with hot methanol almost no further radioactivity could be extracted from livers. The pellet was dissolved in 1 N NaOH, and aliquots were taken for liquid scintillation and for protein determination by the biuret method. Results were expressed as nanomoles of bromobenzene bound per mg of protein.

Major Findings: After administration of a nontoxic dose of ^{14}C-bromobenzene (8 μmoles/mouse) a peak level of 0.2 nmoles of ^{14}C/mg protein bound in the liver was attained within 1 hr and declined slowly with a half-life of about 24 hr. The level of binding increased in proportion to the dose administered and the shape of the curve for the disappearance remained the same for all nonhepatotoxic doses. However, with doses which produced

centrolobular necrosis 45 to 125 μmoles/mouse) the concentration of bound radioactivity continued to increase for 24 hr reaching a peak value of over 10 nmoles/mg protein and then declining with a half-life of about 8 hr. The time course of binding closely paralleled the development of necrotic changes in centrolobular hepatocytes. In contrast to bromobenzene, even a large dose of benzene (113 μmoles/mouse) did not cause necrosis, and the peak value of binding was only 0.5 nmoles/mg at 6 hrs and disappeared from the liver relatively rapidly.

Only these organs rich in cytochrome P-450 microsomal enzymes (liver, kidney, lung) bound significant amounts of radioactivity after ^{14}C-bromobenzene administration, suggesting a relationship between metabolism and binding. More direct evidence of such a relationship was obtained by pretreating mice with phenobarbital (100 mg/kg, i.p. for 3 days) or with phenobarbital + SKF 525-A (75 mg/kg, i.p.). These compounds induce and block, respectively, hepatic microsomal enzymes. The mice were killed 6 hr after receiving 140 μmoles of ^{14}C-bromobenzene, i.p. The amount of bound radioactive material in control livers was 0.35 nmoles/mg compared with 8.30 nmoles/mg in the phenobarbital-treated groups. Injection of SKF 525-A to phenobarbital-treated mice 1 hr before ^{14}C-bromobenzene administration reduced the binding to 0.23 nmoles/mg. Similar results were obtained using ^{14}C-chlorobenzene.

Significance to Biomedical Research and the Program of the Institute: These results confirm our hypothesis that a metabolite of bromobenzene may become covalently bound to liver macromolecules. The temporal correlation of binding with the development of histological evidence of hepatic necrosis, the observation that both the binding and necrosis depend on microsomal enzymes and the autoradiographic evidence (reported elsewhere) that the binding and the necrosis are localized to the centrolobular areas argue for an important relationship between these two phenomena. It is conceivable that covalent binding may play a role in the pathogenesis of a wide variety of chemically-induced tissue lesions, including adverse reactions to drugs.

Projected Course of Project: These studies are being expanded to determine whether similar toxic mechanisms apply to other industrial toxins, carcinogens, air pollutants, food additives and drugs.

Honors and Awards: None

Publications: None

Serial No. NHLI-168
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Possible Explanation for the Zonal Distribution of Chemically-induced Hepatic Necrosis
Previous Serial Number:	None
Principal Investigator:	Dr. Watson D. Reid
Other Investigators:	Dr. Gopal Krishna Dr. Fritz Michel Eichelbaum Mr. John George
Cooperating Unit:	Dr. F.M. Eichelbaum holds a Geigy Fellowship

Project Description:

Objectives: We postulate that the localization of necrosis to a zone of hepatocytes surrounding either centrolobular vein or the portal triad is a reflection of the distribution of drug metabolizing enzymes in the liver lobules. If an enzyme converts its substrate into a highly reactive metabolite capable of alkylating tissue macromolecules it is likely the cells with the greatest enzyme activity would be exposed to the highest concentrations of toxic metabolite and would be the most likely to be damaged. Microsomal metabolizing enzymes are believed to be localized in the centrolobular zone of hepatocytes because the proliferation of smooth endoplasmic reticulum following administration of phenobarbital (an inducer of microsomal enzymes) is limited to these hepatocytes. Furthermore one microsomal enzyme, benzpyrene hydroxylase, has been shown by histochemical techniques to be localized in the centrolobular zone. We have previously shown that the centrolobular necrosis produced by bromobenzene is related to a toxic metabolite, possibly an epoxide, formed by the action of microsomal enzymes. The toxic metabolite becomes covalently bound to liver proteins, and the binding is virtually limited to the centrolobular zone as shown by autoradiography.

In contrast, allyl alcohol, which produces periportal necrosis, is metabolized to the highly reactive aldehyde, acrolein, by alcohol dehydrogenase, a nonmicrosomal enzyme. This enzyme is known to be localized in the periportal zone.

The present studies were undertaken to determine whether allyl alcohol toxicity is produced by a metabolite and whether the parent compound or one of its metabolites becomes tightly bound in the necrotic lesions in the

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peripheral zone.

Methods Employed: Male Sprague-Dawley rats (180 g) were killed 6 or 24 hr after administration of ^{14}C-allyl alcohol. Half of the animals were given pyrazole (375 mg/kg, i.p.) 2 hr before ^{14}C-allyl alcohol in order to block alcohol dehydrogenase in the liver. Paraffin sections of liver were mounted on glass slides and dipped in Kodak NTB-2 emulsion. After exposure for 4 weeks they were developed and stained with hematoxylin and eosin. The remainder of the liver was homogenized in water, precipitated with an equal volume of 20% TCA and the pellet extracted five times with methanol at 60°C. This procedure removed all ^{14}C allyl alcohol added to a homogenate of normal liver.

Major Findings: The periportal necrosis typically produced by allyl alcohol administration (.05 ml/kg, i.p.) was completely blocked by pretreatment with pyrazole. After 5 washings with hot methanol the TCA precipitate from control livers contained .178 and .080 nanomoles of ^{14}C per milligram of protein at 8 and 24 hr, respectively, but the binding was one eighth as great if the animals were pretreated with pyrazole. The autoradiograms revealed that the bound radioactive material was concentrated in the necrotic periportal areas in livers from animals receiving allyl alcohol alone. But no significant binding could be detected by autoradiography after pyrazole administration.

Significance to Biomedical Research and the Program of the Institute: These data suggest that periportal necrosis elicited by allyl alcohol is caused by the binding of a metabolite to hepatocytes. Metabolism, binding and necrosis all occur in periportal zone in the case of allyl alcohol and in the centrolobular area after bromobenzene administration. The three phenomena appear to be related.

Proposed Course of Project: A manuscript is being prepared.

Honors and Awards: None

Publications: None

Serial No. NHLI-169
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Binding and Metabolism of Aromatic Hydrocarbons in the Lung

Previous Serial Number: None

Principal Investigators: Dr. Watson D. Reid
Dr. Gopal Krishna

Other Investigator: Mr. John George

Cooperating Units: None

Project Description:

Objectives: We have expanded our studies on mechanisms to include organs other than the liver. Autoradiograms of tissues of mice injected (i.p.) with ^{14}C-labeled aromatic hydrocarbons such as bromobenzene, chlorobenzene and naphthalene showed a striking accumulation of radioactivity selectively in the bronchial epithelium. The preliminary experiments described in this report provide evidence that a metabolite of ^{14}C-bromobenzene is covalently bound in the lung, and that the lung is capable of metabolizing the hydrocarbon.

Methods Employed: After intraperitoneal administration of ^{14}C-bromobenzene (75-125 μmoles/20 g mouse) the binding of radioactive material in the lung was determined by the same technique as described for the studies in liver. Lung homogenates (saline) were extracted 3 times with heptane, precipitated with 10% TCA, the pellet extracted 5 times with hot methanol, dissolved in NaOH and aliquots taken for counting and for protein determination. The metabolism of ^{14}C-bromobenzene and ^{14}C-chlorobenzene was studied in either the 9,000 x g supernatant or the 105,000 x g microsomal pellet of mouse lung homogenates. The labeled hydrocarbon was incubated for 5 to 30 min in a mixture containing the tissue preparation and a NADPH-generating system. The unmetabolized ^{14}C-bromobenzene was removed by 3 extractions with heptane, and in 1 experiment the heptane phase was washed with NaOH to remove any phenolic metabolites. Aliquots of the aqueous phase were taken for counting and protein determination and the results expressed as nmoles of ^{14}C-bromobenzene metabolized per mg protein per hr.

Major Findings: After administration of ^{14}C-bromo or chlorobenzene to

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mice or rats, approximately one fifth as much radioactivity was bound in the lung as in the liver. The amount of bound material was increased by prior administration of phenobarbital (100 mg/kg, i.p. for 3 days) and inhibited by SKF 525-A, suggesting that the binding was related to the activity of cytochrome P-450 enzymes. In a typical experiment 0.21 nmoles/mg of radioactive material was bound in lung 5.5 hr after ^{14}C-bromobenzene administration to control mice compared with 1.42 nmoles/mg protein in phenobarbital-treated animals. When phenobarbital-treated mice were given SKF 525-A (70 mg/kg, i.p.) 1 hr before administration of the ^{14}C-hydrocarbon the level of binding fell to 0.08 nmoles/mg.

In vitro experiments indicated that both the 9,000 x g supernatant and the 105,000 x g microsomal pellet metabolize ^{14}C-bromobenzene at essentially the same rate. Lung microsomes from control mice metabolized 0.63 nmoles/mg protein/hr and this value was reduced to 0.36 nmoles/mg/hr in the presence of 10^{-4}M SKF 525-A. Surprisingly, the metabolism was not enhanced significantly by using 9,000 x g supernatant or microsomes of lungs from animals treated with phenobarbital. The rate of metabolism using lungs from control animals was about one third to one fourth that in liver, and liver microsomes showed a 3 to 4-fold induction of bromobenzene metabolist after phenobarbital administration. The 9,000 x g supernatant from lung produced considerably more phenolic metabolites than did that of the liver.

Significant to Biomedical Research and to the Program of the Institute: Although bromobenzene induces only minimal damage in the bronchial epithelium, its localization and binding there after parenteral administration is impressive. Although bromobenzene is not believed to be a carcinogen, the possibility that other hydrocarbons might be localized in bronchial epithelium after absorption by some route other than by the respiratory tract could be relevant to the pathogenesis of bronchogenic carcinoma. The data in vitro suggest that the lung may metabolize foreign compounds into reactive intermediates which bind covalently with proteins in the bronchial epithelia. The failure of phenobarbital administration to induce metabolism is inconsistent with this interpretation and raises the possibility that the toxic metabolite is formed in the liver and transported to the lung by the blood stream.

Proposed Course of Project: We plan to study a wide variety of labeled compounds for binding in the lung and look for a correlation between the site of binding and the site of tumor induction. Preliminary experiments are in progress with dimethylnitrosamine, 3,4-benzpyrene, 7,12-dimethylbenzanthracene and thioacetamide. We also intend to determine whether binding occurs in the lung of hepatectomized rats and whether metabolism occurs mainly in epitheleal cells or in alveolar macrophages.

Honors and Awards: None

Publications: None

Serial No. NHLI- 170
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Accumulation of Radioactivity After Incubation of Rat Brain Slices with ^{3}H-choline
Previous Serial Number:	None
Principal Investigator:	Dr. Dean Haubrich
Other Investigator:	Dr. Alan I. Green
Cooperating Units:	Dr. Haubrich holds a Special Fellowship from NIGMS. Dr. Green is a Research Associate LPP:NIMH, St. Elizabeths Hospital, Washington, D.C., NIGMS.

Project Description:

Objectives: Acetylcholine (ACh) is synthesized from choline by the enzyme choline acetyltransferase in the presence of acetyl-CoA. Since the concentration of choline within the brain is near the estimated K_m value of choline acetyltransferase, relatively small changes in choline concentration could alter the rate of ACh synthesis. De novo synthesis of choline does not occur within the brain, but instead choline is taken up from the plasma by an active transport process. Thus the rate of uptake of choline may be an important factor in regulating the synthesis of ACh. In the present study we have examined the uptake of choline and its subsequent conversion to ACh and other choline derivatives in various regions of the brain.

Methods Employed: The accumulation of ^{3}H-choline was examined by a procedure based on that described by Saskin and Snyder (JPET 175: 404-418, 1970). Aliquots of tissue slices (5 mg) of various areas of rat brain were prepared with a tissue chopper and then incubated in Kreb's Henseleit-bicarbonate medium with 2×10^{-8} ^{3}H-choline (choline chloride, methyl-^{3}H, 250 MCi/mM). After ten minutes incubation, the tissue was extracted with 15% 1 N formic acid in acetone. Organic soluble choline derivatives were extracted by the addition of ether. Choline and acetylcholine remaining in the aqueous phase were separated by high voltage electrophoresis.

Major Findings: There were marked variations in the accumulation of

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total tritium in different brain regions. These differences, expressed as tissue to medium ratios (T/M = CPM per gram wet weight of tissue/CPM per ml of medium) were striatum: 12.2; hypothalamus: 5.2; cerebral cortex: 4.2; hippocampus: 3.3; midbrain: 2.7; cerebellum: 0.9. In the striatum about 60% of the radioactivity was accounted for as free choline, 30% as acetylcholine and about 10% as CDP-choline, phosphorylcholine and organic soluble choline derivatives (e.g., lecithin and sphingomeyelin). The percentage of ACh was smaller (10-15%) in the other regions studied. Incubation of the slices in the presence of a cholinesterase inhibitor (Eserine, 10^{-4}M) markedly increased the T/M ratios and the percentage of radioactivity recovered as acetylcholine in all brain areas.

Significance to Biomedical Research and to the Program of the Institute: This study provides the first evidence for a regional difference in the uptake of choline and its conversion to ACh in the brain. We hope that these preliminary findings will help to elucidate some of the basic mechanisms involved in regulating the levels and/or synthesis rates of ACh in the brain.

Proposed Course of Project: The concentrations of choline and ACh and the activities of the biosynthetic and degradative enzyme will be measured in different regions of brain. This information may help to explain the regional difference in choline uptake and ACh synthesis.

Honors and Awards: None

Publications: None

Serial No. NHLI-171
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Studies on the Possible Role of Choline in the Regulation of Acetylcholine Levels in Rat Brain
Previous Serial Number:	None
Principal Investigators:	Dr. Dean Haubrich Dr. Watson D. Reid
Other Investigators:	None
Cooperating Unit:	Dr. Haubrich holds a Special Fellowship from NIGMS

Project Description:

Objectives: Acetylcholine (ACh) is synthesized from choline by the enzyme choline acetyltransferase in the presence of acetyl CoA. De novo synthesis of choline does not occur within the brain (J. Neurochem. 14: 873, 1967) but choline is taken up from plasma (J. Neurochem. 14: 873, 1967) and may be converted directly to ACh or else phosphorylated by choline kinase and then converted to phosphatidylcholine (Biochem. J. 110: 201, 1968). Free choline may subsequently be liberated by the hydrolysis of phosphatidylcholine.

When animals are maintained on a diet deficient in choline, there is an appreciable decrease in the brain level of ACh (J. Nutr. 84:13, 1968), suggesting that the amount of free choline in the brain may regulate the concentration of ACh. This view is supported by the finding that addition of choline to a minced preparation of rat brain produces a significant increase in the level of bound ACh (Can. J. Physiol. Pharmacol. 45: 249, 1967). Moreover, the concentration of free choline within the brain is near the estimated K_m value of choline acetyltransferase, and therefore relatively small changes in the concentration of free choline could alter the rate of ACh synthesis.

The level of free choline within the brain may be regulated by one or a combination of the following possible mechanisms: 1) the rate of uptake of choline into the nerve endings, 2) the rate of conversion of choline to phosphorylcholine (and subsequently to phosphatidylcholine) or 3) the rate of hydrolysis of phosphorylcholine, CDP-choline and phosphatidylcholine to yield free choline. The following experiments represent an initial effort

to elucidate the role of choline in regulation of ACh synthesis and were designed to study 1) the metabolic fate of intravenously injected radiolabeled choline in mice, 2) the incorporation of the choline moiety of phosphatidylcholine into ACh in vitro and 3) the effects of drugs upon the level and/or uptake of choline into the brain.

Methods Employed: Choline and ACh were measured using a new enzymatic assay developed in our laboratory (Project Report Number NHLI-244).

Major Findings: 1) The choline specific activity in the brain of mice (4,000 cpm/nmole measured 30 sec. after injection) declined biphasically following the i.v. injection of ^{3}H-choline (100 μCi), with an initial T-1/2 of 1 minute (measured over the first 15 min) and a longer T-1/2 of about 35 hrs. Radioactivity was quickly incorporated into ACh so that the specific activity reached a plateau between 1 and 4 min (2,000 cpm/nmole), indicating that plasma choline is utilized for ACh synthesis. After 2 hrs, no significant radioactivity could be detected in ACh, despite the relatively high specific activity of choline (500 cpm/nmole), suggesting that the choline fraction having the shortest T-1/2 normally serves as the precursor for ACh.

Radioactivity in the organic soluble choline derivatives (probably phosphatidylcholine) accumulated gradually over the first 6 hrs, after which a plateau was reached and maintained for at least 24 hr.

2) Incubation of phosphatidylcholine-1,2-C-14 with brain homogenates resulted in significant incorporation of radioactivity into ACh, indicating that phosphatidylcholine could potentially contribute its choline moiety to the choline pool involved in ACh synthesis.

3)Several drugs known to interact with cholinergic mechanism were studied for their effect upon brain choline. Pilocarpine was found to raise the level of ACh in brain and also to increase the brain level of intravenously administered radiolabeled choline. Amphetamine, which is known to stimulate ACh turnover, was shown to significantly raise the brain concentration of choline. Both of these findings are consistent with the hypothesis that drugs might alter the level and/or synthesis rate of ACh by influencing the intraneuronal concentration of choline.

Significance to Biomedical Research and the Program of the Institute: ACh is an important neurohumoral transmitter in both the central and peripheral nervous systems. These preliminary findings suggest that the level of free choline may be important in regulating ACh levels and/or synthesis rates, and presumably also the functional activity of cholinergic neurons.

Proposed Course of Project: Efforts will be made to answer the following basic questions: 1) Does phosphatidylcholine serve as a source of choline for ACh synthesis in vivo under conditions when ACh utilization is accelerated? The endogenous stores of phosphatidylcholine will be labeled with choline and the incorporation of radioactivity into ACh will be measured when synthesis is stimulated. 2) Does a change in the concentration of choline and/or choline containing intermediates stimulate ACh synthesis? The accumulation of ACh in homogenates and slices will be measured after the addition of increasing amounts of choline. Similar experiments will be performed in vivo by infusing choline.

Honors and Awards: None

Publications: None

Serial No. NHLI-172
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effect of Prostaglandin E_1 on the EEG and the Levels of Brain Acetylcholine in Rats

Previous Serial Number: None

Principal Investigators: Dr. Dean R. Haubrich
Dr. Watson D. Reid
Dr. Jorge Perez-Cruet

Other Investigators: None

Cooperating Unit: Dr. Haubrich holds a Special Fellowship from NIGMS. Dr. Perez-Cruet is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Prostaglandins are distributed in the central nervous system of various species and are located within subcellular particles of nerve endings. Although their functional role has not yet been fully determined, prostaglandins are released from the cat brain in response to electrical stimulation of the reticular formation, and this release is correlated with EEG arousal (Bradley et al., Brit. J. Pharmacol. 37: 151, 1969). Furthermore, profound sedation, stupor and signs catationia are observed following the intravenous or intraventricular injections of Prostaglandin E_1 (PGE_1) into cats or chicks (E.W. Hirton, Brit. J. Pharmacol. 22: 189, 1964). Since central cholinergic mechanisms are thought to be involved in the EEG arousal reaction (Cuculic et al., Prog. Brain Res. 28: 27, 1968) and in the induction of paradoxical sleep (Khazan et al., Int. J. Neuropharmacol. 6: 279, 1967), we have examined the EEG, muscular tone and brain levels of acetylcholine in rats following the parenteral administration of PGE_1.

Methods Employed: Brain acetylcholine was measured using a new enzymatic assay previously developed in our laboratory (see Project Report Number NHLI-244). The EEG and muscular tone were measured using standard electrophysiological techniques. PGE_1 was administered in two equal doses

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of 1 mg/kg, i.p., given 45 min apart.

Major Findings: The levels of brain ACh measured 90 min after the first dose of PGE_1 were significantly elevated (30%, $P < 0.01$). Furthermore, the animals were asleep and displayed a loss of muscular tone. However, the EEG pattern was characterized by low voltage-high frequency similar to the awake stage.

Significance to Biomedical Research and the Program of the Institute: This study provides the first evidence that PGE_1 induces a state of paradoxical sleep in animals. Since PGE_1 also induces an elevation in brain acetylcholine levels, and was previously shown to increase the turnover of brain serotonin (Reid, Project Report Number 242), it is conceivable that the behavioral effects of PGE_1 are mediated by an interaction with one or both of these neurohumors. This suggestion is consistent with the probable role of serotonin in the induction of sleep (see Jouvet, Adv. Pharmacol. 6B: 265, 1968) and of acetylcholine in EEG arousal and in paradoxical sleep.

Proposed Course of Project: We plan to study the effects of behaviorally inactive prostaglandins upon EEG, levels of acetylcholine and turnover of serotonin. Furthermore, the effect of PGE_1 upon EEG will be determined in rats following the manipulation of serotonin and acetylcholine levels. These experiments should help to establish whether changes in cholinergic and/or serotonergic transmission are related to the induction of paradoxical sleep induced by PGE_1.

Honors and Awards: None

Publications: None

Serial No. NHLI-173
1. Chemical Pharmacology
2. Drug Tissue Interaction
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of Pilocarpine on the Metabolism of Serotonin and Acetylcholine in the Brain

Previous Serial Number: None

Principal Investigators: Dr. Dean R. Haubrich
Dr. Watson D. Reid

Other Investigators: Mr. John W. George

Cooperating Unit: Dr. Haubrich holds a Special Fellowship from NIGMS.

Project Description:

Objectives: Pilocarpine produces a number of excitatory effects upon the central nervous system which in contrast to its peripheral effects, are not antagonized by atropine. The excitatory effects resemble those of strychnine, and are presumed to result from a blockade of central inhibitory mechanisms. Both acetylcholine (ACh) and serotonin probably play an important role in central inhibition, since 1) these neurotransmitters depress the firing of certain neurons and 2) drugs which evoke various types of behavioral inhibitions also alter the activity of central serotonergic or cholinergic systems. The objective of the present study was to assess the effect of pilocarpine upon the metabolism of serotonin and acetylcholine within the brain.

Methods Employed: Acetylcholine levels were measured using a new assay developed in our laboratory (Project Report Number NHLI-244). The synthesis rate of ACh was assessed in vivo by measuring the incorporation of intravenously administered radioactive choline (choline-chloride, methyl-^{3}H) into acetylcholine. Acetylcholinesterase activity was determined by measuring the rate of hydrolysis of radioactive ACh (acetylcholine-chloride, methyl-^{3}H). The turnover rate of serotonin was calculated by multiplying the steady state 5-HIAA concentration by the rate constant of 5-HIAA efflux from the brain after inhibition of monoamine oxidase by pargyline.

Major Findings: Pilocarpine administration (100 mg/kg, i.p.) induced a 50-150% increase in the rat brain levels of ACh. This increase was observed within one hour after drug treatment, persisted for more than 4

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hours, and was not antagonized by atropine pretreatment. It appears that pilocarpine raises the level of ACh by preventing its release from intraneuronal storage sites, since the drug decreased the synthesis of ACh and did not inhibit cholinesterase.

In contrast to its effect upon acetylcholine, pilocarpine markedly stimulated the turnover of serotonin. The duration of the increase in serotonin turnover roughly paralleled the rise in ACh levels and was not antagonized by pretreatment with atropine.

Arecoline, a cholinomimetic whose peripheral effects resemble those of pilocarpine, also stimulated serotonin turnover and increased the level of brain ACh. However, the increase in ACh levels was of shorter duration (less than 10 minutes), was antagonized by atropine pretreatment, and did not parallel the increase in serotonin turnover which was observed one hour after drug treatment.

Significance to Biomecical Research and the Program of the Institute: These results provide the first evidence for a biochemical correlate of the central excitatory effects of pilocarpine, and support the suggestion that both ACh and serotonin are important in central inhibitory mechanisms. Furthermore, our findings suggest a possible interaction between central cholinergic and serotonergic mechanisms in maintaining normal CNS function.

Proposed Course of Project: Pilocarpine may be useful for elucidating the possible mechanisms involved in regulation of ACh synthesis. The rate of uptake of choline into cholinergic neurons may be one important factor for the regulation of ACh synthesis (see new report entitled "Studies on the Possible Role of Choline in the Regulation of Acetylcholine Levels in Rat Brain") and we have discovered that pilocarpine markedly stimulates the uptake of choline into brain. An attempt will be made to determine whether the increase in choline uptake is related to the increase in ACh levels induced by pilocarpine.

Honors and Awards: None

Publications: Page, J.G., Kessler, R.M. and Vesell, E.S.: Strain differences in uptake, pool size and turnover rate of norepinephrine in hearts of mice. Biochem. Pharmacol. 19: 1381-1386, 1970.

Serial No. NHLI-174
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Correlation between beta adrenergic receptor activity and enzyme activity of arterial and cardiac muscle

Previous Serial Number: NHLI-274

Principal Investigators: Dr. Jerome H. Fleisch
Dr. Stanley C. Penzotti, Jr.
Dr. Elwood O. Titus

Other Investigators: Dr. Jay Moskowitz

Cooperating Unit: Drs. Penzotti and Moskowitz are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Adenyl cyclase activity of thoracic and abdominal aorta of young and old rabbits was examined to determine if there was a correlation between adenyl cyclase activity and the physiologically determined beta receptor activity (Fleisch et al., Circ. Res. 26: 151, 1970). No correlation was found. Therefore, an attempt was made to correlate beta adrenergic receptor activity with the activity of other enzymes thought to participate in this pharmacologic receptor system.

Methods Employed: Adenyl cyclase activity of thoracic and abdominal aortas from young and old rabbits was measured in homogenates directly by the method of Krishna et al. (J. Pharmacol. Exp. Ther. 163: 379, 1968) and in intact tissue by a modification of the method of Shimizu et al. (J. Neurochem. 16: 1609, 1969). Ca^{++} ATPase activity was measured by the method of Bader (Biochem. Biophys. Res. Commun. 40: 505, 1970). Phosphodiesterase was measured by the method of Schönhöfer et al. (unpublished observations).

Major Findings: Adenyl cyclase activity of thoracic and abdominal aortas from young and old animals was neither stimulated nor inhibited by isoproterenol, norepinephrine, serotonin, or histamine but was markedly increased by NaF. Based on these data, no correlation could be made between adenyl cyclase activity and the physiologic responses of this tissue. Moreover, only slight Ca^{++} ATPase activity could be demonstrated and it was neither stimulated nor inhibited by isoproterenol. Phosphodiesterase activity was demonstrated in aortic tissue but experiments to correlate its activity with β-receptor activation are still incomplete.

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Significance to Biomedical Research and the Program of the Institute: The correlation of biochemical changes with physiological and pharmacological events in vascular and cardiac muscle may elucidate the mechanisms regulating arterial tone, heart rate, hypertension, etc.

Proposed Course of Project: Further attempts will be made to correlate adrenergic receptor activity with changes in tissue enzyme activity in fractions of tissues suspected of having the highest quantity of pharmacologic receptors.

Honors and Awards: None

Publications:

Schönhöfer, P.S., Skidmore, I.F., Forn, J., and Fleisch, J.H.: Adenyl cyclase activity of rabbit aorta. J. Pharm. Pharmacol. 23: 28-31, 1971.

Serial No. NHLI-175
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Interaction of microtubule protein with colchicine and other antimitotic drugs

Previous Serial Number: NHLI-280

Principal Investigators: Dr. Mark Zweig
Dr. Colin F. Chignell

Other Investigator: Dr. Elwood O. Titus

Cooperating Unit: Dr. Zweig is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: In recent years microtubules have received considerable attention. These subcellular structures are distributed ubiquitously in biological systems but function in the movement of certain cellular components (e.g., chromosomes, axonal granules, pigment granules, thyroid colloid droplets, etc.) and may have a structural role in some systems. The protein subunit of the fibrous microtubule polymer binds colchicine quite specifically. It also forms a crystalline complex with vinblastine.

The microtubule protein provides a good model for studying drug-protein interactions. One goal of this project is to better understand the nature of drug-protein interactions at the molecular level. Since microtubule protein exists in both a polymerized and nonpolymerized (subunit) form and only the latter binds colchicine, this study should yield information relating to the characteristics of the protein and its environment which determine the balance between polymerized and subunit states.

A second goal is to measure the relative strength of binding of various colchicine analogues and other drugs to microtubular protein. The latter is conveniently prepared from mammalian brain which is rich in microtubules. Correlation of the relative binding with the relative effects of these drugs on antimitotic, antitumor and antigout activities may be taken as presumptive evidence that the pharmacological effects of the drugs result from their interaction with microtubules.

Methods Employed: The binding of ^{3}H-colchicine to microtubule protein

was examined by incubating the drug and the protein preparation, adsorbing the protein onto diethyl aminoethyl cellulose filters and then determining protein-bound radioactivity in a scintillation counter. Microtubule protein was prepared from rat brain and used directly as it occurs in the high-speed supernatant, or it was first purified by precipitation from brain supernatant in presence of vinblastine. The affinity of the binding site for various analogues of colchicine was determined by measuring the ability of the latter to compete with ^{3}H-colchicine.

Major Findings: Inhibition of the binding of tritiated colchicine (2.5×10^{-6} M) to brain microtubule protein by 17 derivatives of colchicine (2.5×10^{-5} M) is shown in Table 1. The first seven drugs inhibit quite strongly as a group, while the last 10 are weak or virtually ineffective inhibitors. These first seven drugs also have the greatest in vivo antimitotic or antitumor potency, although colchinol and colchiceine in the second group have some antimitotic/antitumor activity, too. In collaboration with Dr. Maling, we have examined the ability of these derivatives to inhibit Na urate crystal-induced inflammation of the rat paw as a model for gout. In close correlation with the activities stated above, colchicine and derivatives #1-3, 5, 7 were the most potent inhibitors; #4, 6, 8, 9 were intermediate; and the rest were ineffective as inhibitors of rat paw inflammation.

TABLE 1

Compound	% Inhibition
1 Colchicinamide	82
2 N-desacetylthiocolchicine	67
3 Demecolcine	60
4 N-acetyl-iodo-colchinol	59
5 Trimethylcolchicinic acid methyl ether	52
6 N-acetyl-colchinol	50
7 TMCA ethyl ether	39
8 Colchinol	9
9 Colchiceine	7
10 TMCA	1
11 N-benzoyl TMCA	2
12 Iso TMCA methyl ether	4
13 Isocolchicine	2
14 Isocolchiceinamide	1
15 Colchinoic acid	4
16 Colchicoside	3
17 Colchicosamide	2

Several other drugs were examined all at 2.5×10^{-5} M (Table 2).

TABLE 2

Compound	% Inhibition
1 Podophyllotoxin	94
2 Podophyllotoxin isothiouronium bromide	78
3 Vinblastine	25
4 Heparin	92
5 Chloral hydrate	9
6 Aspirin	none
7 Phenylbutazone	"
8 Oxyphenylbutazone	"
9 Indomethacine	"
10 Flufenamic acid	"
11 Promethazine	"
12 Tripellenamine	"

Podophyllotoxin and vinblastine both are antimitotics which interact with microtubules, and both were very effective inhibitors of rat paw inflammation. Heparin and chloral hydrate are two mitotic arresting drugs with C-mitotic activity like colchicine. Drugs #6-10 are anti-inflammatory drugs used clinically against gout. Promethazine and tripellenamine are antihistamines, which were found to be very potent inhibitors of rat paw inflammation.

Significance to Biomedical Research and the Program of the Institute: The molecular basis for drug-protein interactions is in many instances unknown but can be studied with modern physical methods. The interaction between microtubular protein and colchicine is of particular interest, since this ubiquitous protein plays a number of roles in a variety of cells. Occupancy of the colchicine binding site prevents the self assembly into complicated structures which is a striking characteristic of this protein and which is presumably important in the formation of microtubules. These tubules transport norepinephrine storage granules along the axon toward the site of their eventual release. They are present in blood platelets. They are probably responsible for the intracellular transport of lysosomes to the cell surface of polymorphonuclear leucocytes in inflammatory responses. Microtubular protein has similarities to actin and sliding filament models have been proposed for the movement of chromosomes by microtubular structures during mitosis.

Proposed Course of Project: Colchicine binding protein will be isolated and purified from rat brain homogenates using a newly modified procedure that permits the isolation of sufficient quantities for study in about 4 hours. Colchicine protein interactions will be further investigated with optical

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and magnetic resonance techniques. The protein is rich in strongly absorbing aromatic amino acids and colchicine itself has a strong absorption band which is apparently sensitive to changes in the environment. It should therefore be possible to gain information about the nature of the binding site fromoptical rotatory dispersion, circular dichroism and fluorescence studies. A nitroxide spin-labeled analogue of demecolcine has been prepared for electron spin resonance studies of the binding mechanism.

Kinetic studies of the effects of temperature on the rates of binding suggest that conformational adjustment of the protein accompanies the binding process. These studies will be completed.

Honors and Awards: None

Publications: None

Serial No. NHLI-176
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The prevention of isoproterenol tachyphylaxis and the reversal of contractile effects in vascular smooth muscle

Previous Serial Number: NHLI-293

Principal Investigators: Dr. Jerome H. Fleisch
Dr. Elwood O. Titus

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Tachyphylaxis to the smooth muscle relaxing effects of isoproterenol and the consequent appearance of contractile responses in the smooth muscles that have both alpha and beta adrenergic receptors offer potential hazards in the clinical applications of this and related drugs. It is of interest to determine whether drugs can affect the onset of tachyphylaxis and whether they can be used to study the mechanism of this phenomenon.

Methods Employed: Spirally cut thoracic aortic strips from rats and rabbits were prepared by the method of Furchgott and Bhadrakom (J. Pharmacol. Exp. Ther. 111: 265, 1954). Each strip was suspended in a 10 ml isolated organ bath, containing a modified Krebs bicarbonate solution aerated with 95% O_2 and 5% CO_2. Contractions were induced by a variety of agents including angiotensin, serotonin, or norepinephrine and measured isometrically with a Grass FT03 force-displacement transducer. Data were recorded on a Grass polygraph as changes in grams of tension.

Major Findings: Aortae relaxed after administration of isoproterenol, a stimulant of beta adrenergic receptors. A second administration led to a diminished response and subsequent administrations caused the vessel to contract. This phenomenon of tachyphylaxis to the relaxing effects of isoproterenol and "reversal" to give a contractile response was observed in vivo as well as in vitro. The following drugs, when present in the bath fluid, prevented both the tachyphylaxis and the appearance of contractions: Aminophylline, theobromine, caffeine, phentolamine, nitroglycerin, bromo-LSD, papaverine and tetracaine. The minimal concentrations of these drugs necessary for these effects were ascertained.

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Significance to Biomedical Research and the Program of the Institute: Isoproterenol is one of the most frequently used bronchodilators in the treatment of asthma. Its continued effectiveness in a particular case, however, is lost as tachyphylaxis to the beta adrenergic effects develops. It is possible that deleterious effects of continued administration of isoproterenol may result in part from constriction caused by isoproterenol stimulation of remaining alpha adrenergic receptors. The rat aorta offers a useful model system on which the effects of drugs on these mutually antagonistic responses may be tested. If tachyphylaxis and reversal can be prevented in clinical situations, the usefulness of beta adrenergic stimulants would be increased.

Proposed Course of Project: Observations to date suggest that a variety of drugs, mostly organic amines, will prevent the appearance of tachyphylaxis to isoproterenol and that this property cannot be correlated with other effects that these drugs may have, such as the ability to inhibit alpha adrenergic responses, to antagonize serotonin, or to relax smooth muscle by other mechanisms. Comparative studies of the effects of concentration changes on the antitachyphylactic effect and the other properties of these drugs will be continued until this point is clarified.

Honors and Awards: None

Publications: Fleisch, J.H.: Further studies on beta adrenoceptor of rat aorta. Brit. J. Pharmacol., in press.

Serial No. NHLI-177
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The influence of age on the response of smooth muscle to pharmacologic agents

Previous Serial Numbers: NHLI-295 and NHLI-297

Principal Investigators: Dr. Jerome H. Fleisch
Dr. Vaclav Trcka
Dr. Elwood O. Titus

Other Investigators: None

Cooperating Unit: Dr. Trcka is on the staff of The Research Institute of Pharmacy and Biochemistry, Prague, Czechoslovakia

Project Description:

Objectives: To determine the mechanisms responsible for change in drug reactivity of various organs with increasing age. In this study, we have investigated changes with age in the effects of some drugs on target organs in young and older members of various animal species.

Methods Employed: Spirally cut thoracic aortic strips from rats of different age groups were set up according to the procedure described by Furchgott and Bhadrakom (J. Pharmacol. Exp. Ther. 111: 265, 1954). Guinea pig vas deferens were prepared according to Thoa and Maengwyn-Davies (J. Pharm. Pharmacol. 20: 873, 1968). The tissues were suspended in a 10 ml isolated organ bath containing a modified Krebs bicarbonate solution, aerated with 95% O_2 and 5% CO_2. Contractions were measured isometrically with a Grass FT03 force-displacement transducer and recorded on a Grass polygraph as changes in grams tension. The entire tail was excised from Sprague-Dawley rats of known age and the ventral tail artery cannulated with a polyethylene tube. Vascular reactivity was measured by the method of Trcka and Fleisch (unpublished observations).

Major Findings: We previously showed that beta adrenergic receptor activity of rabbit and rat thoracic aorta decreases with increasing age (Fleisch et al., Circulation Res. 26: 151, 1970). Furthermore, alpha adrenergic receptor activity was shown to increase in rat trachea with increasing age (Fleisch et al., Amer. J. Physiol. 218: 596, 1970). We have now shown that the loss in beta receptor activity varies with the strain and source of the animals used.

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Rats of several strains obtained from outside sources (other than NIH) showed a decrease in beta receptor activity with increasing age, but required up to 6 months for complete loss of beta receptor activity. This is in contrast to the 90 days shown to be necessary with NIH rats. Beta receptor activity also decreased in the perfused rat ventral tail artery with increasing age. Efforts to determine whether this smaller vessel sclerosed or suffered a true loss in the ability of the artery to dilate to beta adrenergic stimulation are not yet complete. Finally, preliminary experiments have indicated a loss in alpha adrenergic receptor activity in guinea pig vas deferens with increasing age.

Significance to Biomedical Research and the Program of the Institute: These findings have helped characterize some of the changes with age in the responses of smooth muscle to autonomic drugs. They suggest that mechanisms other than variations in drug metabolism which are usually invoked may explain many of the alterations in pharmacodynamic effects seen with increasing age. These data also have given some insight into the normal changes that might be expected with senescence.

Proposed Course of Project: We plan to characterize the changes in receptor activity with increasing age and to determine if some of the effects of aging can be reversed by treating animals with various drugs.

Honors and Awards: None

Publications:

Schonhofer, P.S., Skidmore, I.F., Forn, J., and Fleisch, J.H.: Adenyl cyclase activity of rabbit aorta. J. Pharm. Pharmacol. 23: 28-31, 1971.

Fleisch, J.H.: Further studies on beta adrenoceptor activity of rat aorta. Brit. J. Pharmacol., in press.

Maling, H.M., Fleisch, J.H. and Saul, W.F.: Species differences in aortic responses to vasoactive amines: The effect of compound 48/80, cocaine, reserpine and 6-hydroxydopamine. J. Pharmacol. Exp. Ther. 176: 672-683, 1971.

Serial No. NHLI-178
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Characterization of the enzymatic ion transport system

Previous Serial Number: NHLI-284

Principal Investigators: Dr. Stanley C. Penzotti, Jr.
Dr. Elwood O. Titus

Other Investigators: None

Cooperating Unit: Dr. Penzotti is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: It has been well established that the Mg^{++} dependent, Na^+-K^+-stimulated ATPase found in cell membranes plays a major role in the transport of monovalent cations into and out of the organ. It seems likely that this transport system is a complicated network of protein, lipoprotein, and phospholipids held in some kind of special arrangement.

Attempts to isolate and purify the ATPase have met with limited success. Through the use of various substrates and inhibitors, however, it is possible to gain some understanding of the sequence of events which take place during the hydrolysis of ATP and the transmembranal movement of cations.

Earlier work in this and other laboratories had suggested that DFP (diisopropylfluorophosphate), an inhibitor of Na^+-K^+ ATPase, may introduce upon hydrolysis a phosphate group at the active center of the enzyme. However, it now appears that the inhibition seen with DFP is due to the F ion released during this hydrolysis. Further investigation of the inhibition by F^- may lead to a better understanding of the functional forms of the ATPase.

Methods Employed: Rat kidney microsomes were prepared by the method of Skou and extracted with NaI solution to remove inactive proteins. Incubations with inhibitors and substrates were conducted under various conditions and the ATPase activity was measured.

Major Findings: It is known from previous work in this and other laboratories that NaF inhibits (Na^+-K^+) ATPase in a manner similar to DFP. The

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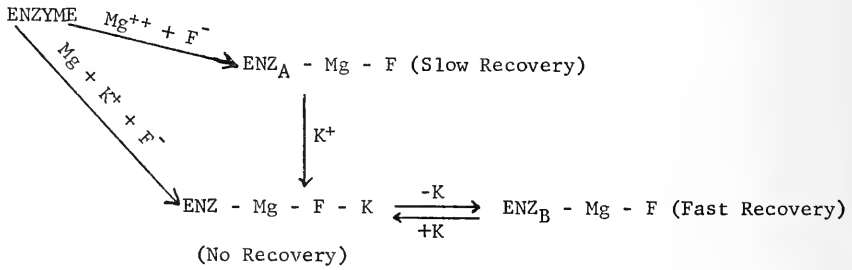
inhibition is dependent on Mg^{++} ion and prevented by the presence of ATP in sufficient concentrations.

The concentration of Mg^{++} for half-maximal inhibition in the presence of fluoride is 0.25 mM. The relationship between Mg^{++} concentration and inhibition does not conform to the usual simple Michaelis-Menten plot but is suggestive of a cooperative effect of Mg. The rate of inhibition of fluoride is greatly enhanced by K^+. The degree of inhibition by fluoride, both in the presence and absence of K^+, varies inversely with pH. It is found in our laboratory that the enzyme remains inhibited after repeated washing at 0-4° and incubation up to 30 min at 37° in Tris buffer (0.1 M, pH 7.4) and 5 mM $MgCl_2$. Recovery after 30 min of incubation in 10 mM EDTA never exceeds 15% above controls. However, incubation of inhibited enzyme with Na_2ATP regenerates the active form. The rate of regeneration is doubled and those enzymes treated with K^+ in addition to the NaF. Exposure of the enzyme which had previously been inactivated by F^- to K^+ for 5 min or less, followed by washing, changes it to the rapidly regenerating form. Reactivation rates plotted as a function of Na^+ concentration are sigmoidal. In 15 mM ATP, the recovery rate is much more sensitive to Na^+ for enzyme inactivated both in the presence and absence of K^+. The reactivation is inversely proportional to K^+ present during treatment with Na_2ATP of the previously inactivated enzyme.

Thus, it would appear that in a K^+-dependent conformation the enzyme reacts rapidly with F^- and the F-enzyme complex can retain a K^+-dependent conformation through several washes as evidenced by the different recovery rates in the presence of Na_2ATP. This can be schematically represented as follows:



Experiments in which Mg^{++} has been replaced with Mn^{++} indicate that the enzyme behaves in a similar way toward F^- inhibition and Na_2ATP recovery.

Significance to Biomedical Research and the Program of the Institute: The cation transport-ATPase system is important in maintaining nervous excitability and is the recovery of sodium by the kidney tubule. The system apparently provides the basis for electrogenic sodium pumps in muscle and various photoreceptors and is important in maintaining the systems that recover norepinephrine in nerve endings and that transport sugars and amino

acids in intestine. The system is the target of cardiac glycoside action in the heart, but the molecular basis for the action of these drugs is not understood.

The formation of stable fluoride complexes by membranous systems may prove to be generally useful, since the adenyl cyclase of brain can be activated for long periods of time whereas the transport ATPase is inactivated. Conformational states induced by cations and preserved in the stable complex can be studied and should offer a clue to the mechanisms of cation activation of membrane enzymes.

Proposed Course of Project: Utilizing the paramagnetic properties of Mn^{++}, we will study further the conformational changes brought about by K^{+} on the F^{-} inhibited ATPase using ESR techniques. Other studies will attempt to isolate and purify the ATPase system through various techniques involving detergents, salts and fractionation. Also attempts will be made to reassemble the ATPase system after disruption by several techniques including application of hydrostatic pressure.

Honors and Awards: None

Publications: None

Serial No. NHLI-179
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Correlation of antiarrhythmic effect of diphenylhydantoin with digoxin-induced changes in contractility, NaK ATPase and K^+ efflux
Previous Serial Number:	NHLI-285
Principal Investigators:	Dr. Robert E. Goldstein Dr. Stanley C. Penzotti, Jr. Dr. Karen S. Kuehl
Other Investigators:	Dr. K. H. Prindle, Jr. Dr. C. A. Hall Dr. E. O. Titus Dr. S. E. Epstein
Cooperating Units:	Drs. Goldstein, Kuehl, Prindle, Hall and Epstein are in the Cardiology Branch, NHLI Dr. Penzotti is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Cardiac glycosides such as digoxin increase the force of contraction of cardiac muscle. These glycosides also selectively inhibit the Na + K-dependent ATPase, which is believed to be responsible for the active transport of Na and K ions. Toxic doses of the cardiac glycosides have been shown to cause an efflux of K^+ from the heart, a consequence compatible with the inhibition of the ATPase system. Diphenylhydantoin has been shown in the clinic to reverse the dysrhythmias induced by digoxin, and this reversal is accompanied by the reversal of the K^+ loss.

The (Na^+-K^+)-dependent ATPase is thus a probable site for the competitive action of diphenylhydantoin to be expressed. In the view of the abundant evidence that the membrane-bound (Na^+-K^+) ATPase is the receptor at which the cardiac glycosides initiate their contractile effect, recent reports that diphenylhydantoin does not reverse the inotropic effects are disturbing. Since the mechanism of digitalis action has been called into question, it was decided to study the effects of diphenylhydantoin on the contractile force of canine myocardium in the absences and presences of digoxin and then measure the effects on the myocardial (Na^+-K^+)ATPase and K^+ efflux under these conditions.

Methods Employed: The right ventricular contractile force was measured by a Walton-Brodie strain gauge in an isolated dog heart perfused in situ with Krebs solution. When the expected drug response was produced, such as the peak contractile strength with digoxin, the heart was immediately perfused with ice-cold Krebs solution and a portion of the left ventricle was removed. Microsomal fractions of the tissue were prepared according to the method of Swartz et al. and the (Na^+-K^+)-dependent ATPase activity was determined. Appropriate samples were taken of the perfusate to determine any changes in K^+ concentration.

Major Findings: Neither control perfusion nor diphenylhydantoin (3×10^{-5} M) alone altered Na^+-K^+ ATPase or produced K^+ efflux. Diphenylhydantoin alone depressed contractile force on average of 29% at 15 min. Digoxin (10^{-6} M) alone in 7 dogs caused a 59% rise in contractile force at the onset of arrhythmias (average 15.0 min of perfusion; ± 0.7 S.E.) along with net K^+ efflux of 50 ± 12 μmoles/min and a decrease in (Na^+-K^+) ATPase from 13.8 to 5.2 μmoles phosphate/mg protein/hr ($P < .001$). Perfusion with diphenylhydantoin and digoxin combined (5 dogs) delayed arrhythmia to 28.9 ± 2.8 min, at which time the contractile force was higher, 91% above control ($P < .05$), K^+ efflux tended to be greater (87 ± 20 μmoles/min) and (Na^+-K^+) ATPase was lower (2.5 μmoles phosphate/mg protein/hr, $P < .05$) than with digoxin alone. Combined diphenylhydantoin-digoxin perfusion which lasted only until the time arrhythmias appeared with digoxin alone (7 dogs), however, yielded higher (Na^+-K^+) ATPase (7.7 S.A., $P < .02$) compared with digoxin alone. K^+ efflux was unchanged while contractile force decreased (26% above control, $P < .05$). Thus, diphenylhydantoin appears to diminish the rate at which digoxin inhibits (Na^+-K^+) ATPase. Nevertheless, diphenylhydantoin ultimately permits digoxin to produce a greater inhibition of (Na^+-K^+) ATPase, a greater increase in contractile force, and a tendency toward greater K^+ efflux than is possible without diphenylhydantoin. This suggests that the antiarrhythmic effect of diphenylhydantoin cannot be totally attributed to prevention of inhibition of Na-K ATPase or to diminution of K^+ efflux, two changes characteristic of digoxin administration.

Significance to Biomedical Research and the Program of the Institute: Although the physical responses of the heart to the cardiac glycosides have been known for some time, the biochemical basis for their effects has not been fully determined. In addition, the nature of the reversal of the digoxin-induced dysrhythmias by diphenylhydantoin is not well understood. The effects of digoxin and diphenylhydantoin on the force of contraction and the (Na^+-K^+)-dependent ATPase led to a better understanding of the mechanisms of action of these drugs as well as the relationship between the ATPase system and its influence on the contractile force.

Proposed Course of Project: Terminated.

Honors and Awards: None

Publications: None

Serial No. NHLI-180
1. Chemical Pharmacology
2. Organic Chemistry
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Synthesis of spin labeled cardiac aglycones for electron spin resonance studies of membrane function

Previous Serial Number: None

Principal Investigator: Dr. Elwood O. Titus

Other Investigator: Mr. Wallace W. Holland

Cooperating Units: None

Project Description:

Objectives: Cardiac aglycones modified by the introduction of stable nitroxide free radicals should be useful in membrane studies. Because of the specificity and stability of the binding of these steroids to those membranous lipoproteins which participate in the transport of cations in nerve, kidney and other tissue, these drugs should provide useful spin labels. The electron spin resonance spectra of bound drug will reflect the extent to which constraints are imposed on the molecular motion of the steroid by its environment. It is anticipated that conformational changes induced by sodium and potassium in a purified transport ATPase from nerve membranes may be revealed by changes in the spectra of bound spin labeled digitalis analogues. There is evidence that, even when inhibited by cardiac glycosides, this enzyme can still undergo cation dependent conformational changes.

An effort is being made to synthesize N-oxyl-4',4'-dimethyloxazolidine derivatives from ketonic aglycones. These derivatives are particularly useful because of their intramolecular rigidity. The orientation of the nitroxyl free radical remains fixed with respect to the steroid nucleus so that the spectra reflect the rotational correlation times of the whole steroid skeleton.

Major Findings: A chromatographically pure spin labeled analogue III of digitoxigenin has been synthesized by the sequence of steps shown below and characterized by mass spectrometry. This synthesis illustrates some of the difficulties yet to be surmounted. These include:

1) Loss of the 14β hydroxyl and formation of a 14,15 double bond which

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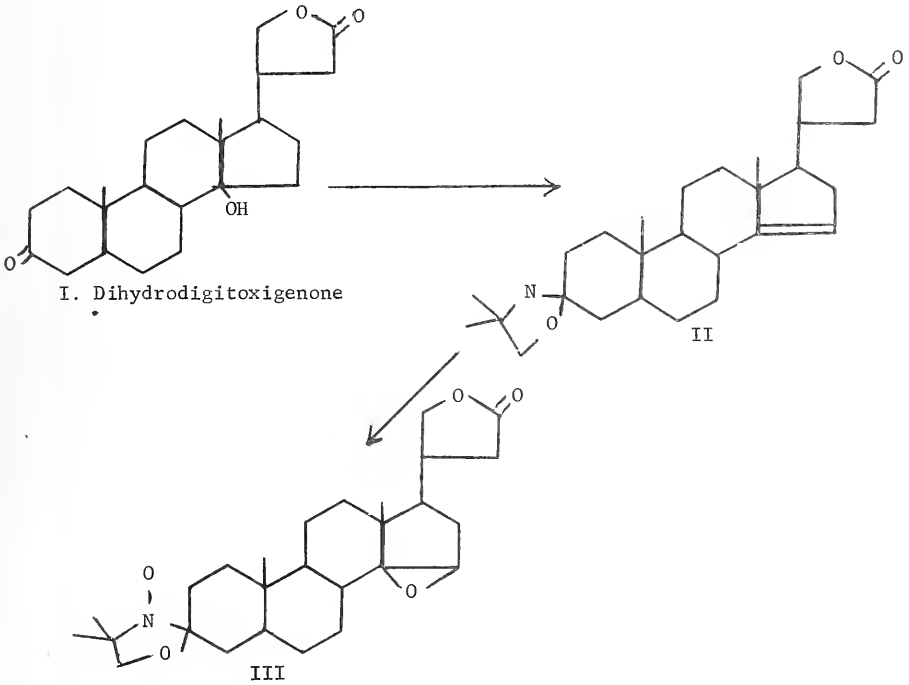
occurs as a side reaction in the ring closure to form the oxazolidine, II.

2) Oxidation of the 14,15 double bond by the reagent (m-chloro-perbenzoic acid) required to oxidize the oxazolidine to a stable nitroxyl radical.

The 3β hydroxyl group lost in the formation of the oxazolidine and the 14β hydroxyl lost by dehydration are both important for the binding of cardiac glycosides to the transport ATPase. Although 14,15 epoxides, e.g. resibufogenin, are known to be biologically active, their activity is less than that of the normally occurring drug, The product, III, was inactive against kidney transport ATPase at 10^{-4} molar, the highest concentration testable.

In a series of experiments to test catalysts that might favor oxazolidine formation without 14,15 dehydration, catalysts of variable acidity caused exactly parallel effects on the two reactions. It would appear that the protonation of oxygen which initiates the two reactions occurs about equally at positions 3 and 14 irrespective of the catalyst.



I. Dihydrodigitoxigenone

II

III

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Significance to Biomedical Research and the Program of the Institute: Because of the remarkable specificity of the cardiac glycosides as inhibitors of the transport ATPase in membrane, they are widely used in studies of the mechanism of membrane transport. If spin labeled analogues can be prepared, these derivatives should become even more useful tools with which to investigate structural changes in membrane that are associated with function.

Proposed Course of Project:

1) Since data to date indicate that unsaturation of the 14,15 position is a necessary accompaniment of the formation of oxazolidine, efforts will be devoted to restoration of the 14β hydroxyl at a later stage in the synthesis. This may be achieved via a 14β hydroxy, 15β bromo derivative with the use of N-bromacetamide and subsequent debromination with Raney Nickel.

2) If reconstruction of the 14 hydroxyl proves practical, introduction of an oxazolidine at the 12 position of digoxigenin (which is sterically hindered so long as 14 is occupied) will be attempted followed by the reintroduction of the 14 hydroxyl. This would give a product bearing the spin label but still retaining those structural features most desirable for binding to the transport site.

Honors and Awards: None

Publications: None

Serial No. NHLI-181
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The mechanism of drug phototoxicity and photosensitivity

Previous Serial Number: None

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Mrs. Donnas K. Starkweather

Cooperating Unit: None

Project Description:

Objectives: Many different drugs, such as the phenothiazines, sulfonamides, tetracyclines, griseofulvin and nalidixic acid, cause photosentivity. Photosensitive reactions can be divided into two main types, namely phototoxic reactions and photoallergic reactions. The phototoxic reaction usually occurs during a patient's first exposure to sunlight after taking the drug and often resembles a sunburn. In contrast, the photoallergic reaction generally does not occur until some time after exposure and may resemble an allergic skin reaction. Photoallergic reactions are often seen at sites which have not been exposed to sunlight.

Although drug photosensitivity is clinically well documented, the precise mechanism of this toxic effect is unknown. We have therefore proposed two possible mechanisms for drug photosensitivity and have begun to test our hypotheses. The first is that photosensitivity results from photodecomposition of these drugs (or their metabolites) into chemically reactive species, such as free radicals and/or carbonium ions, which then react covalently with tissue components. The second is that the energy absorbed by photosensitizing drugs is nonradiatively transferred to biomolecules such as proteins which either undergo decomposition or which become denatured when the energy is converted into the heat of thermal motion. Since the former hypothesis is easier to test, we have studied the effect of light on the stability of a number of drugs known to cause photosensitivity.

Methods Employed: Drugs were dissolved in 0.1 M sodium phosphate buffer or in ethanol and then irradiated in an Aminco-Bowman spectrophotofluorometer. The extent of photodecomposition was measured by recording the fluorescence emission spectrum of each drug as a function of time of exposure to the light

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source. In all cases, the wavelength of irradiation and activation was the same.

Major Findings: Sulfanilamide, a drug which causes marked photosensitivity, showed a 56% decrease in fluorescence intensity after exposure to light at 292 nm for one hour. This decrease was accompanied by the appearance of a marked pink color in the drug solution. Carbutamide, another sulfonamide which is used as a hypoglycemic agent, also showed photodecomposition on exposure to light at 265 nm. Other drugs which exhibited a decrease in fluorescence intensity on irradiation were the antibiotic agent chlortetracycline and the antimalarial chloroquine.

Chlorpromazine showed a marked increase in fluorescence on irradiation at 325 nm over a period of one hour, after which time the fluorescence intensity decreased. Griseofulvin also showed a small increase in fluorescence when irradiated in ethanol solution at 350 nm. The only drug examined which is known to cause phototoxicity but which did not exhibit photodecomposition was nalidixic acid.

Significance to Biomedical Research and the Program of the Institute: If a drug causes photosensitivity, then its therapeutic usefulness is considerably reduced. A knowledge of the mechanism of this phenomenon may permit the design of nontoxic drugs or may suggest a suitable model for testing for this undesirable side effect.

Proposed Course of Project: The photodecomposition of these drugs will be studied in greater detail in an attempt to determine what toxic products are responsible for this phenomenon. An ESR spectrometer equipped with a cavity which can be irradiated by light of different wavelengths will be employed to detect the presence of free radical intermediates. Mixtures of phototoxic drugs and model proteins such as serum albumin will be irradiated in an attempt to detect the formation of covalent bonds between drug and protein.

Honors and Awards: None

Publications: Chignell, C.F.: Fluorescence spectroscopy as a tool for studying drug interactions with biological system. In Chignell, C.F. (Ed.): Methods in Pharmacology, Vol. 2, Physical Methods. New York, Appleton-Century-Crofts, in press.

Serial No. NHLI-182
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of bromobenzene and CCl_4 administration on plasma and hepatic protein synthesis by perfused rat liver

Previous Serial Number: None

Principal Investigators: Dr. Donald C. Davis
Dr. Masahisa Hashimoto

Other Investigators: None

Cooperating Units: Dr. Davis is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Since bromobenzene and CCl_4 produce hepatic centrolobular necrosis, it was of interest to investigate their effects on plasma protein synthesis in an attempt to ascertain the relationship of protein synthesis to bromobenzene-induced liver necrosis. Alterations in protein synthesis in other tissues make *in vivo* results difficult to interpret; therefore, the isolated perfused liver was employed for this study. The perfused liver should be an ideal preparation for such studies, because it allows one to differentiate between alterations in total protein synthesis and other effects.

Methods Employed: A recirculating isolated perfused liver preparation was employed for these studies. Protein synthesis was determined by measuring the incorporation of ^{14}C-leucine into serum and hepatic proteins. Total proteins were separated by trichloroacetic acid precipitation and were counted in a liquid scintillation counter. Protein concentrations were estimated according to standard chemical assay procedures.

Major Findings: Male, Sprague-Dawley rats were treated with 1 ml/kg of either CCl_4 or bromobenzene (i.p., in oil) and killed at either 4 or 12 hours. The livers were perfused *in vitro* with ^{14}C-leucine. Impairment of total protein synthesis (liver + serum) was found at 4 hours after the administration of CCl_4. By contrast, 4 hours after the administration of bromobenzene total as well as serum protein synthesis was higher than in control livers. Twelve hours after bromobenzene pretreatment, total protein synthesis was not

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altered when compared to control livers, but the incorporation of ^{14}C-leucine into serum proteins was decreased. Thus, bromobenzene does not inhibit total protein synthesis, but it may inhibit the release of proteins into the serum.

It was of interest to see if the effect of bromobenzene on protein synthesis could be altered by pretreatment of the animals with several drugs (phenobarbital, SKF-525A, and 3-methylcholanthrene) which are known to affect bromobenzene-induced liver necrosis. Pretreatment of rats with phenobarbital, which increases the severity of bromobenzene-induced necrosis, enhances the effect of bromobenzene to decrease the amount of ^{14}C-leucine appearing in the serum proteins. This effect was evident 4 hours after the administration of bromobenzene, but the effect did not occur until much later (12 hours) when bromobenzene was given alone. Likewise, there was a significant decrease in the incorporation of ^{14}C-leucine into total protein. Thus, the same type of effect as was observed in animals treated with CCl_4 alone was observed in animals which were pretreated with phenobarbital and then given bromobenzene.

SKF-525A and 3-methylcholanthrene (3-MC) prevent bromobenzene-induced liver necrosis. After pretreatment with SKF-525A, bromobenzene not only does not alter total protein synthesis at 12 hours but also does not inhibit the release of proteins into the serum. By contrast, pretreatment with 3-MC does not block the ability of bromobenzene to inhibit the release of proteins into the serum.

Thus, it appears that inhibition of protein synthesis is probably not a primary cause of bromobenzene-induced centrolobular necrosis. The inhibition of release of serum proteins is also probably not involved in the induction of necrosis, since 3-MC blocks necrosis but does not prevent bromobenzene from decreasing the release of proteins into the perfusate.

Significance to Biomedical Research and the Program of the Institute: These studies should help provide a better understanding into the mechanisms involved in drug-induced liver necrosis.

Proposed Course of Project: We shall continue our studies on the mechanisms of drug-induced liver necrosis.

Honors and Awards: None

Publications: None

Serial No. NHLI-183
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The effect of cedrene on drug metabolism by hepatic microsomes

Previous Serial Number: None

Principal Investigators: Dr. Masahisa Hashimoto
Dr. Donald C. Davis

Other Investigators: None

Cooperating Unit: Dr. Davis is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Recent investigations by Wade et al. have shown that the increase in drug metabolism by cedarwood bedding is due to cedrene--the major constituent of cedarwood oil. These authors suggested that the usual route of administration is by inhalation which provides a continuous absorption, although the actual quantity absorbed was assumed to be small.

The present investigation was undertaken to measure the actual dose absorbed via inhalation and to compare the effects of cedrene on hepatic drug metabolism by different routes of administration.

Methods Employed: GLC was employed to measure the concentration of cedrene absorbed by the animals via inhalation. The method consisted of removing samples of air from two reservoirs connected to the inhalation chamber. One reservoir provided an inflow of air into the inhalation chamber while the other reservoir acted as an exit for outflow of air from the chamber. The difference in concentration between the two reservoirs was assumed to be due to absorption of cedrene by the animals, since no difference was found between the two reservoirs when animals were not in the inhalation chamber. The amount of cedrene absorbed was calculated by multiplying the difference in concentration in the two reservoirs by the volume of air flushed through the system during the experiment.

Major Findings: When 50, 100, or 200 mg/kg of cedrene was injected intraperitoneally into female rats once a day for three days, ethylmorphine N-demethylation activity increased 1.6-1.9-fold and cytochrome P-450 content

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increased 1.2-1.5-fold. Oral administration of cedrene (200 mg/kg, once a day for three days) brought about a 2.6-fold increase in ethylmorphine metabolism and a 1.4-fold increase in cytochrome P-450 content. When cedrene was administered by the inhalation route in a dose of 60 mg/kg/day for 2 days or 60 mg/kg/day for 6 days, there was a 1.8-2.2-fold increase in ethylmorphine N-demethylation activity and a 1.3-fold increase in cytochrome P-450 content. There was much less alteration of aniline hydroxylase activity.

The total daily dose of cedrene accumulated during the period of inhalation was found to be comparable to an i.p. dose of 50 mg/kg/day. This is in agreement with the effect of cedrene on hepatic drug metabolism, since the effects were comparable when the drug was administered i.p. or by inhalation. Thus, it appears that the mechanism by which cedrene exerts its effect on the hepatic drug metabolizing system is the same irrespective of the route of administration.

Significance to Biomedical Research and the Program of the Institute: Since a standard method for the quantitaitve measurement of the dose of drugs administered by inhalation has not yet been reported, it is of interest that the present method is useful in studying the pharmacological and toxicological effects of compounds administered by inhalation.

Proposed Course of Project: This project will be terminated.

Honors and Awards: None

Publications: None

Serial No. NHLI-184
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The binding of sulfaphenazole to fetal plasma albumin and to albumin isolated from the plasma of infants and adults

Previous Serial Number: None

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Mrs. Donnas K. Starkweather

Cooperating Units: Dr. Elliot S. Vesell, Department of Pharmacology, Pennsylvania State University, Hershey, Pennsylvania
Dr. Cheston M. Berlin, Childrens Hospital, Washington, D. C.
Dr. E. B. McMahon, Naval Medical Center, Bethesda, Md.

Project Description:

Objectives: Ganshorn and Kurz have reported (Naunyn-Schmiedebergs Arch. Pharmakol. Exp. Pathol. 260: 117, 1968) that the binding of many drugs, including the sulfonamides, is greater in adult plasma than it is in plasma from cord blood. Since the sulfonamides are bound mainly to plasma albumin (J. Pharmacol. Exp. Ther. 129: 282, 1960), we have isolated this protein from cord blood as well as from the plasma of adults and infants and studied its interaction with sulfaphenazole.

Methods Employed: Plasma albumin was isolated by electrophoresis on a starch supporting medium prepared in barbital buffer (0.05 M, pH 8.6) by the method of Kunkel (Meth. Biochem. Anal. 1: 141, 1954). The binding of sulfaphenazole to plasma albumin was measured at 25° by means of equilibrium dialysis.

Major Findings: Serum albumins isolated from eight normal adults had association constants for sulfaphenazole which ranged from $8.2 \times 10^5\ M^{-1}$ to $16.1 \times 10^5\ M^{-1}$ (Table 1) with a mean value of $11.7 \pm 1.0 \times 10^5\ M^{-1}$. This mean value is very close to the association constant of $11.4 \times 10^5\ M^{-1}$ obtained for commercial sample of crystalline human albumin prepared from pooled plasma samples. In contrast, albumins isolated from the plasma of newborns had association constants which ranged from $1.0 \times 10^5\ M^{-1}$ to $12.1 \times 10^5\ M^{-1}$. Although albumin from the youngest infants tended to exhibit

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the weakest binding while the albumin from our oldest infant, an 8-month-old female, had an association constant about the same as an adult, the data are still insufficient to establish a definite change in binding characteristics with time.

Plasma albumins isolated from four samples of cord blood also showed a reduced affinity for sulfaphenazole (Table 2). However, when cord plasma albumin was treated with charcoal, its affinity for sulfaphenazole increased (Table 2). Although Chen has shown (J. Biol. Chem. 242: 173, 1967) that charcoal treatment of plasma albumin will remove bound fatty acids, we have found that other bound anions, such as bilirubin, are also removed by this procedure. When bilirubin was added to a sample of charcoal-treated adult plasma albumin, there was a reduction in the affinity of the protein for sulfaphenazole while the number of binding sites was unchanged (Table 3). Thus, it is quite possible that the differences in the binding properties of plasma albumin isolated from individuals of differing ages is due to the presence or absence of some strongly bound endogenous acidic compound such as bilirubin.

Significance to Biomedical Research and the Program of the Institute: Since newborn infants lack many of the liver microsomal drug-metabolizing enzymes, the binding of a drug to plasma albumin probably represents an important mechanism for removing it from circulation. Furthermore, it has been reported that the administration of acidic drugs, such as the penicillins or the sulfonamides, to newborn infants can displace bound bilirubin from plasma albumin and cause kernicturus. Thus, it is important to know to what extent drugs bind to the plasma albumin of neonates and how such interactions can affect the binding of endogenous ligands, such as bilirubin.

Proposed Course of Project: The physical properties of albumins from cord blood and the plasma of newborn infants will be further studied and their interactions with other acidic drugs measured.

Honors and Awards: None

Publications:

Chignell, C.F.: Physical methods for studying drug-protein binding. In Brodie, B.B. and Gillette, J.R. (Eds.): Handbook of Experimental Pharmacology, Vol. 28, Concepts in Biochemical Pharmacology, Part 1. Berlin, Springer-Verlag, 1971, pp. 187-212.

TABLE 1

The Binding of Sulfaphenazole to Plasma Albumin

No.	Source		Association Constant ($M^{-1} \times 10^{-5}$)	Average number of sulfaphenazole binding sites per molecule
	Sex	Age		
1*	-	-	11.4	0.9
2	M	1 day	2.2	0.5
3	M	3 days	6.3	0.9
4	M	3 weeks	1.0	0.5
5	M	6 weeks	1.6	0.9
6	M	6 weeks	1.0	1.0
7	M	2 months	3.8	0.4
8	M	5 months	4.8	0.4
9	M	8 months	12.1	0.9
10	M	Adult	9.5	0.7
11	M	Adult	9.0	0.6
12	M	Adult	14.0	1.0
13	M	Adult	8.2	0.6
14	M	Adult	11.0	0.4
15	F	Adult	12.2	0.9
16	M	Adult	13.6	0.7
17	M	Adult	16.1	0.5

*Crystalline human plasma albumin purchased from Mann Research Laboratories.

TABLE 2

The Binding of Sulfaphenazole to Cord Plasma Albumin

Plasma Albumin	Association Constant ($M^{-1} \times 10^{-5}$)	Average number of binding sites per albumin molecule
Untreated	6.1	0.9
"	6.1	1.0
"	1.6	0.9
"	5.5	0.7
Charcoal treated	13.0	0.60
"	14.5	0.60
"	12.6	0.65
"	12.9	0.63

TABLE 3

Effect of Bilirubin on the Binding of Sulfaphenazole to Charcoal Treated Human Cord Plasma Albumin

Molar Ratio of Bilirubin to Plasma Albumin	Association Constant ($M^{-1} \times 10^{-5}$)	Average number of Sulfaphenazole Binding Sites per Plasma Albumin Molecule
0	15.8	1.0
0.03	15.1	0.9
0.17	9.5	1.0
0.34	7.2	1.1
1.00	1.3	0.9

Serial No. NHLI-185
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Desoxyisoproterenol: An alpha and beta adrenergic receptor stimulating and blocking agent

Previous Serial Number: None

Principal Investigators: Dr. Jerome H. Fleisch
Dr. Gertrude D. Maengwyn-Davies
Dr. Thaddeus P. Pruss

Other Investigators: None

Cooperating Units: Dr. Maengwyn-Davies is Professor of Pharmacology, Georgetown University Medical and Dental Schools
Dr. Pruss is Director of Pharmacological Research, McNeil Laboratories, Fort Washington, Pa.

Project Description:

Objectives: To study the interactions of an optically inactive analog of isoproterenol, 1-(3,4,dihydroxyphenyl)-2-isopropylaminoethane HCl (desoxyisoproterenol, DOI, isopropyldopamine) with adrenergic receptors of various tissues.

Methods Employed: Spirally cutstrips of rabbit and rat thoracic aorta were prepared by the method of Furchgott and Bhadrakom (J. Pharmacol. Exp. Ther. 111: 265, 1954). Spirally cut tracheal strips were prepared by the method of Constantine (J. Pharm. Pharmacol. 17: 384, 1965). Cat papillary muscles were suspended in 50 ml organ baths and paced using a Grass Model 4 stimulator at a frequency of 1 pulse/sec and a duration of 1 msec at a voltage twice threshold. Cats were prepared according to standard procedure for femoral arterial blood pressure recordings.

Major Findings: DOI was shown to stimulate beta adrenergic receptors in cat papillary muscle, rat aorta and rat trachea in addition to causing a hypotensive effect in anesthetized cats. DOI was also shown to stimulate alpha adrenergic receptors in rat aorta. Furthermore, this compound blocked both alpha and beta receptors in rabbit aortic strips and beta receptors of rat trachea. These different actions indicate that DOI, an analog of isoproterenol which lacks the alcoholic -OH group, exerts both adrenergic receptor stimulating and blocking properties.

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Significance to Biomedical Research and the Program of the Institute: These findings together with others in the literature have helped pinpoint the optimal chemical configuration necessary to interact with adrenergic receptors of various tissues. Furthermore, such findings will ultimately result in the future synthesis of more selective adrenergic receptor agonists and antagonists.

Proposed Course of Project: Terminated.

Honors and Awards: None

Publications: None

Serial No. NHLI-186
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of chronic exposure to atmospheric ozone and of drugs on enzymes

Previous Serial Number: None

Principal Investigator: Dr. Elise Ann Brown

Other Investigator: Dr. Eberhard G. Trams

Cooperating Units: Dr. Trams is with the Laboratory of Neurochemistry, NINDS
National Air Pollution Control Administration, Cincinnati, Ohio

Project Description:

Objectives: Because of its experience with enzyme systems important to heart and brain function, this laboratory is occasionally requested to provide short-term assistance to other agencies. Since ozone is a photochemical byproduct of industrial atmospheric pollutants, the National Air Pollution Control Administration has been interested in the possibility that chronic exposure to O_3 might impair some enzymatic functions in brain.

The Fogarty International Center has requested that we test two new natural products isolated in Pakistan for possible cardiac glycoside-like effects.

Methods Employed: Brain biopsy specimens were obtained from the parietal and occipital lobes of 44 dogs maintained under 5 regimens of ozone exposure for 18 months. These conditions included 1 ppm of O_3 for 8, 16, or 24 hours a day, 2 or 3 ppm of O3 for 8 hours a day and unexposed controls. Standard methods were used for all analyses. Sodium and potassium dependent ATPase was prepared by standard methods in use in this laboratory.

Major Findings: Chronic exposure of dogs to ozone had only minimal effects on any of the components studied. It did not alter either the activities of 5'-nucleotidase and acetylcholinesterase or the formation of lipid hydroperoxides in brain. However, brain monoamine oxidase activity was increased by 19% ($P < 0.01$) in dogs exposed to 1 ppm of ozone for 8 or 16 hours per day but was decreased to 87% of control levels in animals continuously exposed to 1 ppm of O_3. Catechol-O-methyltransferase activity of

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parietal and occipital cortex was decreased to 65% of control levels ($P < 0.01$) in the animals continuously exposed to 1 ppm of ozone. The values of apparent K_m for conventional substrates did not change, suggesting that O_3 caused a loss of enzyme rather than a minor perturbation of structure.

Although the mean catecholamine levels dropped by 35% in animals continuously exposed to 1 ppm of ozone, individual variability in brain catecholamine levels was high and the results were not statistically significant.

The activity of Na^+-K^+-activated ATPase did not change significantly after any of the exposures to O_3. However, there was a small but significant ($P < 0.01$) decline in the Mg^{++}-activated ATPase in animals exposed to 2 and 3 ppm of O_3.

Two new substances, corosin, $C_{30}H_{46}O_7$, and saxin, a terpenic lactone, $C_{14}H_{16}O_9$, have been isolated from plant materials in Pakistan. At the request of the Fogarty International Center, these were tested for cardiac glycoside-like activity with negative results.

Significance to Biomedical Research and the Program of the Institute: Ozone is highly reactive with unsaturated lipids. If sufficient ozone were to reach the brain during chronic exposure, it might cause damage to the membrane bound enzymes there in addition to the bronchitis, pneumonitis and emphysema now known to occur.

Proposed Course of Project: Terminated.

Honors and Awards: None

Publications: None

Serial No. NHLI-187
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Detection of Conformation Shifts in Cell Membranes during Normal and Altered Function by the use of Electron Spin Resonance

Previous Serial Number: NHLI-287

Principal Investigator: Dr. Elise Ann Brown

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Since the components of biological systems are rarely paramagnetic, the paramagnetic resonance spectrum of a stable free radical introduced into a biologically important molecule may be used to gain structural information. The shape of the spectrum will depend on the immediate environment of the radical. Marked broadening of the spectral bands occurs if the ability of the radical to tumble freely in space is restricted by viscosity or by the constraints of adjacent groups on a protein surface. Stable organic radicals (spin labels), usually cyclic nitroxides, can be attached to groups that either react to form covalent bonds with specific sites on proteins or bind strongly to specific regions, such as hydrophobic area of membrane proteins. The spectrum of the spin label may then be used as an indicator of the conformational status of the eceptor site.

Since magnetic resonance techniques, unlike optical spectroscopy, can be applied to particulate or membrane-bound structures, spin labels offer promise as a means of probing for conformational changes associated with the function of (Na^+-K^+) dependent ATPase. This enzyme participates in the transport of Na^+ and K^+ through cell membranes. Recent studies have been concerned with the use of a spin labeled analogue of the substrate, ATP.

Methods Employed: The rates of release of phosphate from ATP by sodium and potassium dependent enzymes were measured by conventional methods. Electron paramagnetic resonance spectra were determined on a Varian E-4 spectrometer.

Major Findings: Adenosine 5'diphosphate-4-(2,2,6,6-tetramethyl-piperidinyl-1-oxyl)(ADP-spin label) was prepared by the method of Weiner and

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new separation techniques were devised to purify this compound. The ADP spin label is not a substrate for the (Na + K) dependent ATPase from rat kidney since no degradation products could be detected by thin layer chromatography after 30 min of incubation with kidney enzyme at 37°. The compound is, however, an effective inhibitor of transport ATPase, 0.2 mmolar giving 50% inhibition. The interaction between ATPase and the inhibitor must be complex, since Dixon plots of reciprocal activity vs inhibitor concentration are not linear and a fraction of the enzyme (about 25%), although normally responsive to sodium and potassium, is resistant to inhibition by the spin label.

Significance to Biomedical Research and the Program of the Institute: The transport of cations through membranes maintains osmotic balance in all cells and excitability in nerve and muscle. Transmembrane sodium gradients are also regulators of sugar, amino acid and catecholamine transport in various tissues. Since the transport enzymes are membrane bound and active only in insoluble complexes, physical methods suitable for studies of mechanism are desirable.

Proposed Course of Project: Efforts to explain the apparent two populations of resistant and inhibitable transport ATPase will continue. Greater concentration of enzyme activity will be necessary to achieve significant ESR changes with an inhibitor that is approximately 50% bound at 0.2 mM. Purification efforts will continue.

Honors and Awards: None

Publications: None

Serial No. NHLI- 188
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Physicochemical studies of complexes between drugs and biomolecules. V. Interaction of anionic drugs with acetylsalicylic acid treated human serum albumin

Previous Serial Number: NHLI-277

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Mrs. Donnas K. Starkweather

Cooperating Unit: None

Project Description:

Objectives: Hawkins and co-workers have shown that acetylsalicylic acid can acetylate a specific lysine group in human serum albumin (HSA) both in vivo and in vitro (Science 160: 780, 1968; J. Clin. Invest. 48: 536, 1969). Since these workers also found that acetylsalicylic acid treated HSA bound more acetrizoate (3-acetylamino-2,4,6-triiodobenzoate), it was of interest to determine whether acetylation altered the interaction of other anionic drugs with HSA.

Methods Employed: Circular dichroism measurements were made with a Cary 60 spectropolarimeter. Acetyl-C^{14}-salicylic acid was synthesized from acetic-C^{14}-anhydride and salicylic acid.

Major Findings: We have previously shown that acetylation of HSA by ASA increased the affinity of the protein for phenylbutazone but decreased its affinity for flufenamic acid. The structural changes in HSA caused by acetylation were reflected by alterations in the extrinsic Cotton effects generated by the binding of phenylbutazone and flufenamic acid to the protein. In order to determine whether these structural changes represented a major conformational change in HSA, the intrinsic optical activity of the protein was measured after incubation alone, with ASA, or with salicylic acid. Results showed that neither the strong negative circular dichroism band exhibited by HSA at 222 nm (due to the α-helical portions of the protein) (Table 1) nor the optical activity of the protein at higher wavelengths (due to tyrosine and tryptophan) was affected by acetylation. This suggests that acetylation of HSA by acetylsalicylic acid does not cause gross changes in the conformation of the protein but causes instead a small

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localized perturbation at or near binding sites for both phenylbutazone and flufenamic acid. The specificity of this interaction is shown in Table 2, where it can be seen that when acetylsalicylic acid is replaced by equimolar concentrations of other acylating agents, such as acetic anhydride, benzylpenicillenic acid, or benzylpenicillin, there is no change in the extrinsic optical activity of phenylbutazone subsequently added to the HSA.

Since a reduction of the incubation temperature from 37° to 25° abolished the effect of acetylsalicylic acid on the extrinsic Cotton effect generated by the binding of phenylbutazone to HSA (Table 1), it became of interest to determine whether the protein was still being acetylated under these conditions. HSA was therefore incubated with acetyl-C^{14}-salicylic acid. Our results showed that when the incubation temperature was reduced from 37° to 25° the incorporation of C^{14}-acetyl into HSA decreased by about 70% (Table 3). When flufenamic acid was included in the incubation medium, there was no reduction in the labelling by acetyl-C^{14}-salicylic acid while phenylbutazone caused a 14% reduction in labelling.

These results suggest (a) that acetylation of HSA by acetylsalicylic acid does not cause a major change in the conformation of the protein, and (b) that the site of acetylation is not the binding site of either phenylbutazone or flufenamic acid. Possibly acetylation at some other site causes a small local perturbation of the protein which is sufficient to alter the binding sites for flufenamic acid and phenylbutazone.

Significance to Biomedical Research and the Program of the Institute: The fate of a drug in the body is greatly dependent upon its interaction with plasma proteins such as albumin. For many drugs, especially those which are very insoluble at physiological pH, the plasma proteins play an important transport function and make it possible for the blood stream to carry drugs in concentrations far exceeding their normal solubilities. The binding of a drug to plasma albumin also affects its free concentration in the plasma and may reduce its availability to metabolizing enzymes in the liver. Thus, if acetylsalicylic acid can modify the affinity of HSA for other drugs, then it may profoundly alter their rate of metabolism and excretion.

Proposed Course of Project: The reaction of acetylsalicylic acid with HSA will be studied in greater detail in an effort to find out what factors affect the rate and course of this acetylation. Other drugs which are esters will also be examined to determine whether they can also acylate HSA.

Honors and Awards: None

Publications:

Chignell, C.F. and Starkweather, D.K.: Optical studies of drug protein complexes. V. The interaction of phenylbutazone, flufenamic acid and dicoumarol with acetylsalicylic acid treated human serum albumin. Mol. Pharmacol., in press.

Chignell, C.F. and Starkweather, D.K.: Species differences in the binding of phenylbutazone to human plasma albumin. Pharmacology, in press.

TABLE 1

Molar ellipticity at 222 nm of human serum albumin

Human serum albumin (1×10^{-4} M) was incubated with drug (5×10^{-4} M), where indicated, then dialyzed at 4° against 0.15 M NaCl and finally distilled water.

Addition	Incubation		Molar Ellipticity $[\theta]_{222} \times 10^{-7}$ (deg.cm^2 decimole^{-1})
	Time (hr)	Temperature (°C)	
None	0	--	-1.41
None	24	37	-1.40
Acetylsalicylic acid	24	37	-1.43
Salicylic acid	24	37	-1.40

TABLE 2

Molar ellipticity of phenylbutazone bound to human serum albumin

Human serum albumin (1×10^{-4} M) was incubated with drug (5×10^{-4} M), indicated, then dialyzed at 4° against 0.15 M NaCl and finally distilled water. Phenylbutazone was then added to the protein and its extrinsic optical activity measured.

Addition	Incubation		Molar Ellipticity (at 287 nm) $[\theta]^* \times 10^{-3}$ (deg.cm^2 decimole^{-1})
	Time (hr)	Temperature (°C)	
None	0	--	+ 53.9
None	24	37	+ 53.7
Acetylsalicylic acid	24	37	+ 75.8
Acetylsalicylic acid	24	25	+ 52.4
Salicylic acid	24	37	+ 51.4
Benzylpenicillenic acid	24	37	+ 53.0
Benzylpenicillin	24	37	+ 52.8
Acetic anhydride	24	37	+ 53.2

*Calculated with respect to the concentration of bound drug.

TABLE 3

Incorporation of C^{14}-acetyl from C^{14}-acetylsalicylic acid into human serum albumin

Human serum albumin (4×10^{-4} M) was incubated with C^{14}-acetylsalicylic acid (1×10^{-3} M) in the presence of 0.02 M sodium phosphate buffer, pH 7.4.

Addition	Incubation		Moles of C^{14}-Acetyl incorporated per mole of HSA
	Temperature (°C)	Time (hr)	
None	37	2	0.14
None	37	4	0.24
None	37	6	0.28
None	37	24	0.61
None	25	24	0.18
Phenylbutazone (2×10^{-4} M)	37	24	0.54
Flufenamic acid (")	37	24	0.61
Dicoumarol (")	37	24	0.65

Serial No. NHLI-189
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Physicochemical studies of complexes between drugs and biomolecules. VIII. The interaction of spin-labelled sulfonamides with bovine erythrocyte carbonic anhydrase B

Previous Serial Number: None

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Mrs. Donnas K. Starkweather

Cooperating Unit: None

Project Description:

Objectives: Spin labels are free radicals which are stable in aqueous systems at physiological pH values and which can be used as reporter groups or labels for biological molecules. The most commonly employed spin label is the nitroxide free radical (=N-O) which can be used in two different ways to study interactions with biological systems. Firstly, the nitroxide group can be attached (covalently or noncovalently) to a macromolecular component of biological system. Sandberg and Piette (Agressologie 9: 59, 1968) have used this approach to study the interaction of chlorpromazine with erythrocyte ghost membranes by labelling the latter covalently with a maleimide nitroxide. A second approach to this problem is to build the nitroxide group into the drug molecule and then to study the fate of the spin-labelled drug molecule in an appropriate biological system. We have chosen this second approach to study the interaction of a spin-labelled sulfonamide with bovine carbonic anhydrase B.

Methods Employed: Bovine carbonic anhydrase B was purified from a commercial preparation by passage over a DEAE cellulose column (Biochim. Biophys. Acta 39: 218, 1960). Spin-labelled sulfonamide I (n = 0) was prepared by condensing p-carboxybenzene sulfonamide with 4-amino-1-oxyl-2,2,6,6-tetramethylpiperidine in tetrahydrofuran containing N-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (J. Amer. Chem. Soc. 90: 1651, 1968). Spin-labelled sulfonamide II (n = 0) was prepared in similar fashion from sulfanilamide and 3-carboxy-1-oxyl-2,2,5,5-tetramethyl-pyrrolidine. Electron spin resonance (ESR) spectra were recorded at ambient temperature with a varian E-4 spectrometer using an aqueous sample cell. The hydrolysis of p-nitrophenylacetate by carbonic anhydrase was measured by observing the

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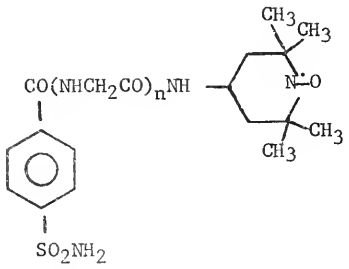
increase in optical density which occurred 348 nm.



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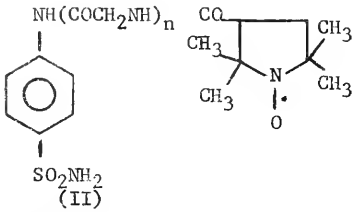

(II)

Major Findings: Both spin-labelled sulfonamides noncompetitively inhibited the hydrolysis of p-nitrophenylacetate by bovine erythrocyte carbonic anhydrase. The K_i for sulfonamide I (n = 0) was 1.2×10^{-6} M. While the noncompetitive nature of this inhibition may seem surprising, since it is known that sulfonamides bind to the active site of carbonic anhydrase, similar results have been reported for acetazolamide, a potent inhibitor of the enzyme (J. Biol. Chem. 241: 5137, 1966). The apparent noncompetitive nature of this interaction may be due to a relatively slow rate of release of the inhibitor.

Both sulfonamide inhibitors quenched the native tryptophan fluorescence of bovine carbonic anhydrase. Fluorescence quenching titration indicated that there was 0.9 mole of sulfonamide I (n = 0) bound per mole of enzyme. The association constant for this interaction was 1×10^{6} M^{-1}. Sulfonamide II (n = 0) was more weakly bound to the enzyme which made it difficult to estimate its binding parameters with any degree of accuracy.

The ESR spectrum of sulfonamide I (n = 0) when free in solution consisted of three narrow lines which resulted from hyperfine interaction between the unpaired electron of the nitroxide group and the nucleus of the nitrogen atom. In the presence of bovine carbonic anhydrase, the spectrum of I

(n = 0) resembled the rigid glass, powder, or polycrystalline spectrum of the nitroxide radical. This result suggests that when I (n = 0) binds to bovine carbonic anhydrase B the nitroxide group becomes immobilized at the enzyme binding site. When ethoxyzolamide, a drug which has a very high affinity for carbonic anhydrase, was added to the spin label-enzyme complex, the sulfonamide was displaced from its binding site and once more exhibited an ESR spectrum characteristic of a free molecule.

X-ray crystallographic studies of human carbonic anhydrase C containing the inhibitor acetoxymercurisulfanilamide have indicated that the inhibitor occupied a deep narrow crevice (J. Mol. Biol. 25: 505, 1967). If this picture has any validity for bovine carbonic anhydrase B, it is perhaps not surprising that the nitroxide group of sulfonamide I (n = 0) became immobilized when the drug bound to the enzyme.

Significance to Biomedical Research and the Program of the Institute: Erythrocyte carbonic anhydrase catalyzes the hydration of carbon dioxide and the formation of bicarbonate ion. It represents one of the few instances where the "receptor" for a drug molecule has been isolated, purified, and characterized to such an extent that in vitro studies of drug interactions are possible. Spin labelling is a relatively new technique which will undoubtedly become of great importance for studying drug mechanisms.

Proposed Course of Project: The topography of the bovine carbonic anhydrase will be explored by means of analogs of I and II in which n = 1, 2, etc. When the number of glycyl groups is increased, the ESR spectrum of the sulfonamide spin label should eventually indicate that the nitroxide group is no longer interacting with the binding site. Thus, it will be possible to estimate the depth of the active site of bovine carbonic anhydrase B.

Spin label derivatives of other drug molecules, including the barbiturates, local anesthetics and certain choline inhibitors of acetylcholinesterase, will be synthesized and their interaction with biological systems studied. When used in conjunction with immunological techniques, such derivatives may also be useful for the detection and assay of small amounts of drugs in biological fluids.

Honors and Awards: None

Publications:

Chignell, C.F.: Spectroscopic techniques for studying the interaction of drugs with biological macromolecules. Advan. Drug Res. 5: 55-94, 1970.

Serial No. NHLI-190
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Physicochemical studies of complexes between drugs and biomolecules. VII. The use of fluorescent probes to monitor drug interactions with red cell ghost membranes

Previous Serial Number: None

Principal Investigator: Dr. Colin F. Chignell

Other Investigator: Mrs. Donnas K. Starkweather

Cooperating Unit: None

Project Description:

Objectives: Feinstein and co-workers (FEBS Letters 6: 245, 1970) have shown that when 1-anilinonaphthalene-8-sulfonic acid (ANS) binds to bovine erythrocyte ghost membranes the fluorescence yield of this probe increases 50-fold while its fluorescence emission maximum shifts from 520 nm to 470 nm. When the membranes were perturbed by calcium ions or the anesthetic butacaine, the fluorescence of bound ANS increased. Since these workers believed that ANS was bound to the phospholipids of the ghosts, they suggested that calcium ions and butacaine perturbed the lipid portion of the membrane.

Recently, Waggoner and Stryer (Proc. Nat. Acad. Sci. 67: 579, 1970) have reported the synthesis of several fluorescent probes and studied their interaction with phosphatidyl choline vesicles. From their results they inferred that the fluorescent moieties of 12-(9-anthroyl)-stearic acid (AS), N-dansyl phosphatidyl ethanolamine (DPE), and N-octadecyl naphthyl-2-amino-6-sulfonic acid (ONS) probed the hydrocarbon, glycerol and aqueous interface regions, respectively, of the phospholipid bilayer membrane. We have therefore used these same probes to study the interaction of butacaine and other membrane perturbing drugs with human erythrocyte ghost membranes.

Methods Employed: Human erythrocyte ghost membranes were prepared by the method of Dodge et al. (Arch. Biochem. Biophys. 100: 119, 1963). Fluorescence was measured in an Aminco Bowman Spectrophotofluorometer and was corrected for self absorption.

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Major Findings: The fluorescence emission maximum of AS bound to human erythrocyte ghost membranes indicated that the anthroyl group of the probe was present in a hydrophobic region of the membrane. In contrast, the dansyl group of DPE was probably in a region close to the membrane-water interface, since its fluorescence emission maximum when bound to the membranes was almost the same as when the probe was dissolved in water. The fluorescence emission maximum of ONS indicated that the fluorescent moiety of this probe was present in the aqueous phase.

When butacaine sulfate was added to membranes containing bound AS, the fluorescence intensity of the probe decreased while its fluorescence emission maximum was unchanged. In contrast, the fluorescence of membrane-bound DPE or ONS was unaffected by the addition of butacaine. Tetracaine, a local anesthetic structurally related to butacaine, caused a decrease in the fluorescence of membrane-bound AS and DPE while not affecting the fluorescence of ONS. These results suggest that, while butacaine perturbs only the hydrophobic regions of the human erythrocyte ghost membrane, tetracaine causes a more widespread change in membrane structure.

Flufenamic acid, an anti-inflammatory agent which stabilizes erythrocyte membranes at low concentrations ($< 10^{-5}$ M) but labilizes them at high concentrations ($> 10^{-4}$ M), did not affect the fluorescence of membrane-bound AS but decreased greatly the fluorescence of DPE. Preliminary results suggest that this effect may be due to displacement of DPE from the membrane.

TABLE 1

The Fluorescence Emission Maximum of AS in Organic Solvents and Human Erythrocyte Membranes

Solvent	Fluorescence Emission Maximum (nm)
n-Hexane	445
Benzene	455
Ethanol	470
Methanol	470
Erythrocyte ghost membranes	445

TABLE 2

The Fluorescence Emission Maximum of DPE in Organic Solvents and Human Erythrocyte Membranes

Solvent	Fluorescence Emission Maximum (nm)
Hexane	475
Benzene	482
Ethanol	515
Water	520
Erythrocyte ghost membranes	515

TABLE 3

The Fluorescence Emission Maximum of ONS in Organic Solvents and Human Erythrocyte Membranes

Solvent	Fluorescence Emission Maximum (nm)
n-Butanol	412
Methanol	415
Methanol-water (3:1)	420
Water	430
Erythrocyte ghost membranes	430

Significance to Biomedical Research and the Program of the Institute: It is becoming increasingly apparent that many drugs exert their pharmacological effect by interacting with cell membranes. The erythrocyte ghost membrane is affected by many of these drugs and therefore provides a useful system for studying their mechanism of action. Once the mechanism of a drug is fully understood at a molecular level, it becomes possible to design more effective therapeutic agents.

Proposed Course of Project: The interaction of other drugs, known to modify membrane function, with human erythrocyte ghost membranes will be studied.

Honors and Awards: None

Publications: Chignell, C.F.: Fluorescence studies of drug interactions with biological systems - A review. Fluorescence News 5: 1-5, 1970.

Serial No. NHLI-191
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Hepatotoxicity of Halogenated Aryl Hydrocarbons. 3. Pulse Label Studies on Bromobenzene Metabolism

Previous Serial Number: None

Principal Investigators: Dr. David Jollow
Dr. Nicola Zampaglione

Other Investigators: Dr. Maynard Hamrick
Dr. Bitten Stripp
Dr. James R. Gillette

Cooperating Units: Drs. Maynard Hamrick and Nicola Zampaglione are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Prior studies have indicated that protection from bromobenzene hepatotoxicity in rats, afforded by 3-methylcholanthrene pretreatment, is associated with an increased bromobenzene dihydrodiol formation. The present studies were undertaken to use urinary metabolite profiles as an estimate of relative activities of epoxide S-glutathione transferase and epoxide hydrase in vivo, at short time intervals after bromobenzene administration.

Methods Employed: 1. Glutathione disappearance: Glutathione depletion from the liver cells was measured after an intraperitoneal injection of bromobenzene (0.2 ml/200 g rat). 2. Pulse studies: Rats were injected with bromobenzene i.p. (0.2 ml/200 g rat) and subsequently ^{14}C-bromobenzene (2 uc) dissolved in 0.5 ml of plasma was injected into the femoral vein at time intervals of 0, 1.5, 3, 6, 9 and 12 hr after i.p. administration. Urine was collected for three hr after the i.v. injection.

Major Findings: 1. At short time intervals (0-3 hr), mercapturic acid was the major metabolite (80%) in normal rats. The ratio of bromocatechol + bromobenzene dihydrodiol to mercapturic acid, reflecting the distribution of the epoxide into the epoxide hydrase and epoxide S-glutathione transferase pathways was 0.09. At longer time intervals the proportion of mercapturic acid in the metabolites decreased, consistent with the depletion of GSH from the cells. The ratio of the hydrase to transferase pathways

increased but never exceed 0.3. 2. The rate of depletion of GSH from the liver of 3-methylcholanthrene induced rats was similar to that found for untreated rats. 3. At all time intervals the formation in 3 MC induced rats of mercapturic acid was decreased and the formation of the dihydrodiol and catechol was increased. The ratio of epoxide hydrase mediated pathways to epoxide S-glutathione transferase mediated pathway was 1.0 at short time intervals decreased only to 0.65 at longer time intervals. 4. These data suggest that 3-methylcholanthrene induces epoxide hydrase relative to epoxide S-glutathione transferase and possibly to bromobenzene epoxide synthetase activity. Increased hydrase activity, particularly after glutathione depletion from the liver cell, would act to decrease the steady state level of bromobenzene epoxide and hence to decrease arylation of cellular macromolecules and the subsequent necrosis.

Significance to Biomedical Research and the Program of the Institute: These studies emphasize that a variety of factors in drug metabolism may influence the toxic manifestations of drug therapy and that these factors may be influenced by prior exposure to drugs or other environmental conditions.

Proposed Course of Project: Studies on the mechanism of bromobenzene metabolism will be continued with emphasis on the variety of metabolic pathways and the factors influencing the distribution of the epoxide intermediate into these pathways.

Honors and Awards: None

Publications: None

Serial No. NHLI- 192
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on Drug-Induced Hemolytic Anemia

Previous Serial Number: None

Principal Invesiigators: Dr. David Jollow
Dr. James R. Gillette

Other Investigators: Dr. Jerry R. Mitchell
Dr. Amin Suria

Cooperating Units: Dr. Mitchell is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Hemolytic anemia associated with glucose-6-phosphate dehydrogenase deficiency is an example of a drug-induced lesion associated with a genetic trait of man. Although a variety of clinical evidence is available that drug metabolism is a necessary prerequisite for hemolytic anemia, little is known about the nature of the metabolites, how they interact with the red blood cell and whether or not the metabolites exert their toxic effects by covalent binding to cell constituents. A major handicap in these studies has been the lack of a suitable *in vitro* or *in vivo* model system (apart from man himself). The present studies were aimed at examining parameters useful in assessing drug damage to the red blood cell and in the capacity of liver slices to provide metabolites for study. Attention has been given to the capacity of epiandrosterone to inhibit red blood cell glucose-6-phosphate dehydrogenase and hence mimic the human deficient red blood cell.

Methods Employed: 1. Drug interaction with red blood cells has been followed using the methylene blue decolorization test, glutathione depletion, Heinz body formation, methemoglobin formation, heme liberation and other hemoglobin modifications. 2. Liver slices were incubated at 37° with acetanilid and sulphanilamide for 30 min and suspending fluid was then transferred and incubated with RBC to assess metabolite-RBC interaction.

Major Findings: 1. Epiandrosterone-inhibited rat, rabbit and human (normal) red blood cells (RBC) mimic human glucose-6-phosphate dehydrogenase deficient cells when challenged with acetylphenylhydrazine as shown by the methylene blue decolorization test, a marked depletion of glutathione, heme loss, Heinz body formation and cell hemolysis. 2. Attempts to implicate

metabolism of acetanilid and sulfanilamide by liver slices with the hemolytic effects have not been successful, possibly due to tight binding of metabolites to liver slice proteins.

Significance to Biomedical Research and the Program of the Institute: Glucose-6-phosphate deficient hemolytic anemia is a major complication of drug therapy, particularly in antimalarial treatment, for both negro and mediterranean caucasian populations. Understanding of the mechanism of toxicity is essential for drug therapy and for drug design.

Proposed Course of Project: The project will be continued to examine other methods of obtaining drug metabolism and to examine the effects of the metabolites and of acetylphenylhydrazine on epiandrosterone-inhibited RBC and on human glucose-6-phosphate deficient cells.

Honors and Awards: None

Publications: None

Serial No. NHLI-193
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Inhibition by Colchicine Derivatives and other Antimitotic Agents of Rat Hindpaw Edema Induced by Sodium Urate Crystals

Previous Serial Number: None

Principal Investigators: Dr. Mark Zweig
Dr. Harriet M. Maling
Dr. Marion E. Webster

Other Investigators: Mrs. Martha A. Williams
Mr. William Anderson, Jr.

Cooperating Units: Dr. Zweig is a Research Associate in the Pharmacology-Toxicology Program, NIGMS
Dr. Webster and Mr. Anderson are members of the Experimental Therapeutics Branch, NHLI

Project Description:

Objectives: The specific binding of colchicine to microtubule protein has been proposed (Malawista, Arth. Rheum. 11:191, 1968) as a mechanism by which this drug exerts antigout and antimitotic activity. Direct evidence for the binding of colchicine to the microtubule protein of the spindle and concomitant disruption of the mitotic apparatus has been reported in the literature, but there is no clear demonstration of an involvement of microtubules in the alleviation of acute gouty arthritis. This project is a study of the ability of colchicine, its derivatives and other mitotic agents to inhibit the edema induced in the rat's hindpaw by sodium urate crystals. These findings are correlated with the in vitro binding to microtubule protein (Zweig and Chignell, 1970-71 Project Report) and the in vivo antimitotic, antitumor and antigout activities of these agents reported in the literature.

Methods Employed: As a model for gouty inflammation, the edema produced in the rat's hindpaw after subplantar injection of sodium urate crystals was used as previously described (Webster and Maling, Adv. Exp. Med.

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Biol. 8:493, 1970). Drugs were administered subcutaneously in three separate equal doses at 0, 2 and 4 hr after the subplantar injection of 10 mg crystalline sodium urate. Six hours after the initiation of the experiment, the paws were amputated and the inflammation is expressed as the difference in weight of the control paw (injected with 0.1 ml saline) and that receiving the urate crystals. The dose of a drug which inhibited 50% of the inflammation (ID_{50}) was determined from dose-response curves.

Major Findings: The ability of the various compounds to inhibit the urate-induced edema is given in Table 1. Also included is their ability to compete in vitro with colchicine for binding to rat brain microtubule protein (data taken from 1970-71 Project Report by Zweig and Chignell). Colchicine at 2 mg/kg suppressed almost all (71-87%) of the inflammation induced by the urate crystals. Certain other derivatives of colchicine (N.2-5) appeared to be equally effective. When dose-response curves for colchicine and derivatives No.2,3, and 5 were determined, the ID_{50} values, calculated in μmoles/kg, were found to be remarkably similar, all lying between 1.0 and 2.6 μmoles/kg.

Four other derivatives of colchicine (No.6-9) were less potent (Table 1). Two of these No.6 and 7), when tested at 10 mg/kg, gave inhibition similar to that of colchicine at 2 mg/kg. Two other derivatives (No. 8 and 10), even at this high dosage, inhibited the edema by only 58 and 28%, respectively. TMCA (No.11) was tested only at 10 mg/kg and was found to be ineffective. The remaining derivatives (No.12-18) did not inhibit the urate-induced edema when tested at 2 mg/kg and their action was not investigated further. It appeared, clear therefore, that the derivatives of colchicine could be grouped into three classes, those as good as colchicine (No.2-5), those of only moderate effectiveness (No.6-10) and those without effect.

Three other drugs (Table 1) which have been shown to bind microtubule protein and interact with microtubular structures were tested for their ability to inhibit urate-induced edema. Two of these, podophyllotoxin (19) and vinblastine (21), produced good inhibition, but PTB (20) had no effect, possibly because this drug is not absorbed at physiological pH.

As shown in Table 1, those derivatives most capable of binding to the microtubule protein in vitro, with the exception of N-acetyl-iodo colchinol, are those which also inhibit the rat-paw edema induced by sodium urate crystals. A review of the literature on the antimitotic and antitumor activities of these derivatives would suggest that these activities also correlate with the above antiinflammatory activity. If the protein subunit is similar in diverse microtubule systems, as is generally believed, these data indicate that microtubules or microtubule protein is involved in disruption of the mitotic spindle, inhibition of inflammation and binding to a brain protein.

Table 1. Inhibition by colchicine derivatives and other antimitotic compounds of rat hindpaw edema induced by urate crystals.

No.	Compound	% Inhibition at 2 mg/kg*	% Inhibition at 10 mg/kg*	ID_{50} µmoles/kg	% Inhibition of ^{3}H-colchicine binding**
1	Colchicine	87,71		1.0	-
2	Demecolcine	87,86		1.2	60
3	Colchiceinamide	78,75		1.9	82
4	TMCA[a] methyl ether	78,86		2.6	52
5	TMCA ethyl ether	79		NT	39
6	DTC[b]	50,43	80		67
7	N-acetyl colchinol	26,46,29	75		50
8	Colchiceine	43,(23),(9)	58		7
9	Colchinol	45,(19),(21)	NT		9
10	N-acetyl-iodo colchinol	(42),(11),(26)	28		59
11	TMCA	-	(26)		1
12	Colchicosamide	(18)			2
13	Colchicoside	(2)			3
14	Isocolchicine	(7)			2
15	Iso TMCA methyl ether	(16)			4
16	Isocolchiceinamide	(0)			1
17	N-benzoyl TMCA	(0)			2
18	Colchinoic acid	(0)			4
19	Podophyllotoxin	64			94
20	PTB[c]	(0)			78
21	Vinblastine	67			25

**Taken from 1970-71 Project Report by Zweig and Chignell.
* All values are significantly different from saline controls except those in parentheses.
N.T. - Not tested because of insufficient material.
a) TMCA = trimethylcolchicinic acid
b) DTC = desacetylthiocolchicine
c) PTB = podophyllotoxin isothiouronium bromide

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Significance to Biomedical Research and the Program of the Institute: Only a few of these compounds have been tested clinically in acute gout. Colchicine, demecolcine, TMCA and vinblastine were reported to be effective, colchicoside gave equivocal results, and colchiceine was ineffective. Our data agree with the observation that colchicine, demecolcine, and vinblastine are effective anti-gout agents, but would class TMCA, colchicoside and colchiceine as ineffective. Also, the data suggest that three other derivatives, TMCA methyl ether, TMCA ethyl ether and colchiceinamide, deserve further investigation, with the hope that one of these compounds might be less toxic than colchicine, demecolcine or vinblastine.

Proposed Course of Project: A manuscript is now in preparation for publication.

Honors and Awards: None

Publications: None

Serial No. NHLI-194
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effects of Colchicine and Antagonists of Histamine and Serotonin on Inflammation Induced by Sodium Urate Crystals

Previous Serial Number: NHLI-135

Principal Investigators: Dr. Harriet M. Maling
Dr. Marion E. Webster
Dr. Mark Zweig

Other Investigators: Mrs. Martha A. Williams
Mr. William Anderson, Jr.

Cooperating Units: Dr. Zweig is a Research Associate in the Pharmacology-Toxicology Program, NIGMS
Dr. Webster and Mr. Anderson are members of the Experimental Therapeutics Branch, NHLI

Project Description:

Objectives: Our earlier investigations (NHLI-135) had shown that subplantar injection of sodium urate crystals into the rat's hindpaw produced an inflammation which was only partially inhibited by antagonists to the kallikrein-kinin system. Further insight into the underlying mechanism of this edema was sought by comparing the ability of colchicine, ten antihistaminesand two serotonin antagonists to reduce this urate-induced inflammation.

Methods Employed: Inflammation was produced by subplantar injection of sodium urate crystals (10 mg/paw). Saline (0.1 ml) was injected into the other paw. Six hours later, when the inflammation was maximal, the paws were amputated at the ankle. The inflammation is expressed as the difference in weight of the hindpaws.

Major Findings: Colchicine has been known to be effective in clinical gout for more than a century and gouty inflammation is thought to be due to the deposition of sodium urate crystals. If rat hindpaw edema induced by sodium urate crystals is a satisfactory experimental model of gouty inflammation, the edema should be suppresed by treatment with colchicine. As

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shown in Table 1, colchicine (2 mg/kg) when injected subcutaneously at 0, 2 and 4 hr after the injection of sodium urate crystals almost completely prevented the edema. In two different experiments, colchicine produced 87 and 78% inhibition.

The mechanism responsible for the effectiveness of colchicine does not appear to depend on its ability to inhibit the inflammation induced by endogenous mediators such as serotonin, histamine or bradykinin, since colchicine more effectively inhibited the edema induced by sodium urate crystals than that produced by these mediators (Table 1). Also, colchicine only partially inhibited the edema induced by Compound 48/80, an edema resulting from the release of serotonin and histamine from mast cells (Maling, Webster and Zweig, Project Report). The effectiveness of colchicine probably resides in the ability of this compound to bind to microtubule protein as shown in other studies (Zweig, Maling and Webster, Project Report).

Table 2 compares the inhibition produced by large doses of two serotonin antagonists and ten antihistamines of rat hindpaw edema induced by sodium urate crystals. Since neither of the serotonin antagonists, cyproheptadine and methysergide, inhibited the sodium urate edema, serotonin probably does not participate in this type of inflammation. Histamine may be playing a role since eight of the ten antihistamines significantly reduced the urate-induced inflammation. However, two antihistamines, chlorcyclizine and diphenhydramine, failed to inhibit significantly this edema. The observation that two of the antihistamines, promethazine and tripelennamine, were able to inhibit the urate-induced edema as effectively as colchicine (Compare Table 1 with Table 2) suggests that these compounds may be effective antagonists of gouty inflammation. When dose-response curves for these two antihistamines were determined, the ID_{50} values, calculated in μmoles/kg, were similar (54 for promethazine and 34 for tripelennamine).

Significance to Biomedical Research and the Program of the Institute: These data suggest that the subplantar injection of sodium urate crystals into the rat's hindpaw is a suitable experimental model for gouty inflammation. Bradykinin and possibly histamine may be playing a role in this inflammation, but serotonin does not appear to be involved. Two of the antihistamines, promethazine and tripelennamine, in large doses could suppress urate inflammation as effectively as colchicine and deserve further investigation as possible anti-gout agents.

Proposed Course of Project: Further investigation into the mechanism of sodium urate edema may be made and the inhibition of this edema by carboxypeptidase B will be confirmed under conditions in which it clearly inhibits inflammation induced by bradykinin. A manuscript will be prepared.

Honors and Awards: None

Publication: Webster, M.E. and Maling, H.M.: Evidence for and against the kinins as endogenous mediators of arthritis. Adv. Exp. Med. Biol. 8: 493-501, 1970.

Table 1. Inhibition by colchicine of edema induced by sodium urate crystals, serotonin, histamine, bradykinin and Compound 48/80.

Edema induced by	% Inhibition
Sodium urate, 10 mg[a]	87*,78*
Serotonin, 1 µg	37*
Histamine, 200 µg	3
Bradykinin, 100 µg	35*
Compound 48/80, 10 µg	30*

Colchicine (2 mg/kg) was injected subcutaneously at 0, 2 and 4 hr after the injection of sodium urate crystals (10 mg); edema was measured at 6 hr. In experiments with the other agents, inflammation was induced at 5 hr and measured 1 hr later.

* Significant inhibition, $p < .05$.
[a] Dose per paw.

Table 2. Inhibition of edema induced by sodium urate crystals by two serotonin antagonists and ten antihistamines.

Compound[a]	% Inhibition
Serotonin antagonists	
Methysergide	15
Cyproheptadine	9
Antihistamines	
Promethazine	76*
Tripelennamine	73*
Antazoline	55*
Pyrilamine	52*
Chlorpheniramine	48*
Phenindamine	46*
Carbinoxamine	45*
Triprolidine	44*
Chlorcyclizine	32
Diphenhydramine	26

[a] Methysergide (1 mg/kg), cyproheptadine (4 mg/kg) and the antihistamines (50 mg/kg) were injected subcutaneously at 0,2 and 4 hr after the injection of sodium urate crystals (10 mg); edema was measured at 6 hr.

* Significant inhibition, $p < .05$.

Serial No. NHLI-195
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Correlations Between the Effects of Hepatotoxic Compounds on Plasma and Liver Triglyceride Levels and the Development of Hepatic Necrosis
Previous Serial Number:	None
Principal Investigators:	Dr. Harriet M. Maling Dr. Benjamin Highman
Other Investigators:	Mr. Wilford Saul Mrs. Martha A. Williams
Cooperating Unit:	Dr. Benjamin Highman is PHS Liaison Officer and Chief, Radiopathology Division, Armed Forces Institute of Pathology. He is also a member of the Laboratory of Experimental Pathology, NIAMD.

Project Description:

Objectives: It is well-known that a fall in plasma triglycerides and triglyceride accumulation in the liver precede the development of hepatic necrosis after the administration of carbon tetrachloride. Other compounds, such as thioacetamide and bromobenzene, also produce liver necrosis, but the effects of these agents upon liver and plasma triglycerides have not been studied. Scattered comments in the literature, based on histologic studies, suggest that liver triglyceride accumulation may not occur after the administration of bromobenzene or thioacetamide. We have measured plasma and liver triglyceride levels at various times after the administration of hepatotoxic agents, in a search for possible correlations between decreased plasma triglyceride levels, elevated liver triglyceride levels, and the development of hepatic necrosis. We have also measured liver glycogen levels and plasma levels of glucose and FFA, as indicators of glycogen and fat mobilization. The effects of phenobarbital induction of drug-metabolizing enzymes and pretreatment with various blocking compounds will be determined, to see whether potentiation or inhibition of triglyceride accumulation in liver is associated with potentiation or inhibition of necrosis.

Methods Employed: Plasma levels of glucose, FFA, and triglycerides and liver content of triglycerides and glycogen are measured by standard methods.

After fixation in buffered formalin, paraffin sections are stained with azur-eosin for the evaluation of necrosis. Frozen sections are stained for neutral fat with oil red O. Additional sections of liver are fixed in acetic acid-alcohol-formalin and stained with periodic acid-Schiff reagent for the detection of glycogen.

Major Findings: Liver triglyceride content in female rats increased from about 4 mg/g to 12-15 mg/g in the first six hours after the administration of bromobenzene (0.50-1.0 ml/kg, i.p.). Frozen sections stained with oil red O showed that the fat accumulated primarily in the centrolobular region. Liver triglycerides did not increase significantly after the administration of bromobenzene to male rats. However, plasma triglycerides fell in both male and female rats, beginning about 8 hr after the administration of bromobenzene and decreasing at 24 hr to levels about 50% those in control rats.

In female rats, thioacetamide (150 mg/kg, i.p.) increased the liver triglyceride content four fold in 18 hr, even though liver triglycerides were unchanged at 6 hr. Necrosis and fat accumulation were centrolobular. Plasma triglycerides fell to about 40% control levels in 6 hr and were still low at 18 hr. Liver triglycerides in female rats were already significantly elevated 6 hr after the administration of allyl alcohol (0.05 ml/kg) and increased to about 23 mg/g at 18 hr. Frozen liver sections stained with oil red O showed that the triglycerides were mainly deposited in the periportal region. Plasma triglycerides were slightly decreased at 6 hr (77% control values), but were higher than controls at 18 hr. Periportal necrosis was evident as early as 6 hr after the administration of allyl alcohol.

Bromobenzene, allyl alcohol, and thioacetamide all caused elevated plasma FFA and glucose levels in female rats at 6 hr. Glycogen depletion at 18 hr was marked, about 95% after thioacetamide and almost 98% after allyl alcohol. As early as 6 hr after allyl alcohol, glycogen depletion was 80%.

These measurements indicate that mobilization of fat and glycogen occur early after the administration of allyl alcohol, bromobenzene and thioacetamide. Triglycerides accumulate in the liver and plasma triglycerides fall; these findings suggest an impairment of hepatic release of triglycerides, possibly the result of impaired synthesis of protein.

Bromobenzene, administered in small doses (0.15 and 0.50 ml/kg, i.p.) to rats which has been treated with phenobarbital (80 mg/kg, i.p. for three days) failed to produce significant triglyceride accumulation in livers of either female or male rats. These small doses of bromobenzene decreased plasma triglyceride levels markedly in rats pretreated with phenobarbital, but only slightly reduced plasma triglyceride levels in control rats. The phenobarbital treatment itself increased the levels of plasma triglycerides in control rats killed 6 hr after injections of sesame oil (1.0 ml/kg). The failure of small doses of bromobenzene to induce significant triglyceride accumulation in livers of rats pretreated with phenobarbital is in marked

contrast to the potentiation of hepatic necrosis which has been reported from this laboratory by Brodie et al (Proc. Nat. Acad. Sci. 68:160-164, 1971). This finding suggests that hepatic necrosis is not always preceded by triglyceride accumulation in liver.

Triglyceride accumulation in liver induced by bromobenzene (0.75 and 1.0 ml/kg, i.p.) was completely prevented by pretreatment with Dibenamine (50 mg/kg, s.c. 24 and 48 hr before bromobenzene). We have previously reported that Dibenamine treatment also prevents triglyceride accumulation in livers of female rats after CCl_4 and ethionine (Maling et al., Fed. Proc. 19:229, 1960). Experiments are now in progress to determine the effects of Dibenamine treatment on the development of necrosis and on the fall in plasma triglyceride levels produced by bromobenzene.

Significance to Biomedical Research and the Program of the Institute: This project should unravel some of the confusion about the relationships between decreased plasma triglyceride levels, elevated liver triglyceride levels, and liver necrosis. Our findings to date suggest that the combination of decreased plasma triglycerides and elevated liver triglyceride levels in female rats is usually associated with the development of hepatic necrosis.

Proposed Course of Project: We are planning to study the effects of various treatments which have been reported to block either triglyceride accumulation in liver or hepatic necrosis. We have previously reported that Dibenamine blocks both triglyceride accumulation and necrosis in the liver induced by subcutaneous administration of CCl_4 (Butler et al., Fed. Proc. 19: 229, 1960). Other investigators have reported that certain antihistamines (e.g., promethazine)blocks necrosis induced by CCl_4 without preventing fat accumulation. These interrelationships require further study.

Honors and Awards: None

Publications: Krawczak, J.J. and Brodie, B.B.: Effect of adrenalectomy and complete blockade of adrenergic function on mortality from histamine, endotoxin, formalin and tourniquet stress in rats. Pharmacology 3: 65-75, 1970.

Serial No. NHLI-196
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Antihistamines and Inflammation Induced by Histamine, Serotonin, Bradykinin and Compound 48/80

Previous Serial Number: NHLI-288

Principal Investigator: Dr. Harriet M. Maling
Dr. Marion E. Webster

Other Investigators: Mrs. Martha A. Williams
Mr. William Anderson, Jr.

Cooperating Unit: Dr. Webster and Mr. Anderson are members of the Experimental Therapeutics Branch, NHLI

Project Description:

Objectives: There has been much speculation concerning the roles of various endogenous mediators in acute inflammation of the rat's paw produced by a variety of phlogogenic agents. Many investigators have been skeptical of a possible contribution of histamine to rat hindpaw edema induced by a variety of agents because: 1. subplantar injection of histamine, even in very large doses, produces little edema; and 2. several antihistamines have been reported to be relatively ineffective against some types of inflammation. In these studies we have compared the ability of 10 antihistamines and 3 serotonin antagonists to inhibit the edema induced by 3 endogenous mediators, histamine, serotonin and bradykinin. These data are used to evaluate the roles of the various endogenous mediators in inflammation induced by Compound 48/80.

Methods Employed: The compound producing the inflammation was injected into the left hindpaw and saline into the right hindpaw. One hr after injection, each rat was killed by decapitation and the paws were amputated at the ankle. Inflammation was measured as the difference in weight of the hindpaws. The antihistamines and serotonin antagonists were injected s.c. 15 min before injection of the phlogogenic agent subplantarly into the hindpaw. Suspensions of rat peritoneal mast cells were used in in vitro studies with these compounds.

Major Findings: Dose-response curves for the three endogenous mediators, histamine, serotonin and bradykinin, when compared to the dose-response curve for Compound 48/80 (assume molecular weight of 516), showed that serotonin and Compound 48/80 were approximately equipotent, that six times as much

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bradykinin was required to produce the same inflammation, and that 100 times as much histamine produced only half the inflammation. Although histamine was much less potent than serotonin in producing edema, the paw contains much more histamine than serotonin, about 40-50 ug/paw histamine in contrast to 0.3 ug/paw of serotonin.

The hypothesis that histamine release contributes to the inflammation induced by Compound 48/80 is supported by our finding that large doses of a number of antihistamines, effectively antagonize the edema. The seven antihistamines in Table 1 which inhibit both serotonin and histamine effectively suppress the edema induced by Compound 48/80. However, significant inhibition of the edema induced by Compound 48/80 is also produced by three antihistamines, triprolidine, carbinoxamine, and chlorpheniramine, which do not significantly inhibit serotonin edema.

A combination of 2-bromo-LSD, which effectively antagonizes serotonin without modifying the response to histamine, and chlorpheniramine, which antagonizes histamine, but not serotonin, almost completely prevents the inflammation produced by Compound 48/80, as shown in Table 2.

The contribution of serotonin alone to the edema induced by Compound 48/80 can be estimated from the percent inhibition achieved with the two most specific serotonin antagonists, 2-bromo-LSD and methysergide (Table 1). About two-thirds of the edema is apparently the result of serotonin release by Compound 48/80. The higher inhibition achieved with cyproheptadine is probably associated with other factors, such as its significant antagonism against bradykinin and the antihistamine activity which has been reported by others.

Four compounds, promethazine, antazoline, diphenhydramine, and cyproheptadine, were also capable of significantly reducing the edema induced by bradykinin. In our 1970 report (NHLI-288), we stated that the kinins probably do not play a major role in the inflammation induced by Compound 48/80, since the edema was not reduced significantly by pretreatment of the rats with soybean trypsin inhibitor or carboxypeptidase B., substances which are capable of preventing kinin formation or destroying the formed kinin.

Carbinoxamine, phenindamine, and particularly tripelennamine appeared to be more effective against Compound 48/80 than can be explained by their effectiveness against either serotonin or histamine. In an effort to explain this discrepancy, the compounds were tested at concentrations of 2×10^{-3}M on suspensions of peritoneal mast cells to estimate both 1. their ability, if any, to release serotonin and histamine from the mast cells, and 2. their effectiveness in preventing release of these mediators by Compound 48/80 (8 ug/ml). Promethazine, phenindamine, and chlorcyclizine could by themselves cause release of histamine and serotonin from mast cells. On the other hand, tripelennamine, triprolidine, chlorpheniramine and diphenhydramine inhibited most markedly the release of these mediators from the mast

cells by Compound 48/80. No correlation could be seen between these two activities and the ability of these compounds to inhibit the edema induced by Compound 48/80.

Significance to Biomedical Research and the Program of the Institute: Many persons suffer from acute and chronic inflammation of various types. This project should increase our understanding of mechanisms involved in inflammation. A study of the effectiveness of various compounds against experimental inflammations may lead to the clinical trial of promising compounds. Effectiveness against edema induced by endogenous mediators should be of special interest.

Proposed Course of Project: Carboxypeptidase B will be retested for its ability to inhibit inflammation induced by Compound 48/80, under conditions in which it clearly inhibits inflammation induced by bradykinin.

The combination of a specific histamine antagonist, triprolidine, and methysergide, the most specific serotonin antagonist known, will be tested for its ability to suppress completely the edema induced by Compound 48/80.

Honors and Awards: None

Publications: Severs, W.B., Gordon, J.W., Beaven, M.A. and Jacobsen, S.: Some observations on aminoguanidine pharmacology. Pharmacology 3: 201-208, 1970.

Table 1. Inhibition of edema induced by Compound 48/80 (10 ug/paw) by pretreatment of rats with histamine and serotonin antagonists.

Compound[a]	% Inhibition of Edema			
	48/80	Serotonin	Histamine	Bradykinin
Compounds which antagonize both serotonin and histamine				
Tripelennamine	93*	42*	80*	16
Pyrilamine	90*	47*	97*	0
Promethazine	88*	78*	84*	46*
Antazoline	87*	61*	41*	32*
Diphenhydramine	67*	62*	63*	24*
Phenindamine	57*	38*	38*	26
Chlorcyclizine	57*	36*	72*	14
Compounds which inhibit histamine, but not serotonin				
Triprolidine	61*,38*	3	74*	1
Carbinoxamine	56*	15	24	0
Chlorpheniramine	26*	4	36*	0
Compounds which inhibit serotonin, but not histamine				
Cyproheptadine	84*	62*	27	34*
2-Bromo-LSD	69*	95*	23	15
Methysergide	55*	95*	16	0

[a] Each compound was injected in a dose of 50 mg/kg s.c. except for cyproheptadine (4 mg/kg), 2-bromo-LSD (2 mg/kg), and methysergide (1 mg/kg)

* $p < .05$

Table 2. Suppression of edema induced by Compound 48/80 (10 ug/paw) by the combination of a specific serotonin antagonist, 2-bromo-LSD (10 mg/kg i.p.) and a specific antihistamine, chlorpheniramine (50 mg/kg s.c.)

Antagonist	g. edema ± S.E.	% Inhibition
Saline	.678 ± .030	Control
2-Bromo-LSD	.185 ± .030	73
Chlorpheniramine	.537 ± .030	21
2-Bromo-LSD + Chlorpheniramine	.074 ± .033	89

Serial No. NHLI-197
1. Chemical Pharmacology
2.
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Salivation in Mice as an Index of Adrenergic Activity.

Previous Serial Number: NHLI-290 (1969-1970

Principal Investigator: Dr. Harriet M. Maling
Dr. Theodore Koppanyi

Other Investigators: None

Cooperating Units: Dr. Koppanyi is Professor of Pharmacology at Georgetown University School of Medicine and Dentistry

Project Description:

Objectives: The data accumulated during the two years, 1968-1970, has been analyzed and is now being incorporated into manuscripts suitable for publication. The relationships between adrenergic salivation and body temperature are being critically evaluated.

Methods Employed: Salivation and temperature responses to sialogogues and antisialogogues were measured at ambient temperatures ranging from 4° to 42°C.

Major Findings: It was found that the effectiveness of both sialogogues and blocking agents is dependent on the ambient temperature. Small doses of d-amphetamine, norepinephrine, epinephrine and l-isoproterenol produced greater salivation and temperature responses at ambient temperatures of 30-32° than at room temperature. The alpha adrenergic blocking agent, phentolamine, blocked completely the salivation induced by d-amphetamine and l-isoproterenol at room temperature, but reduced only slightly the salivation induced at elevated temperatures. Since phentolamine was relatively ineffective in blocking salivation responses to norepinephrine and epinephrine at room temperature, it was not tested against these sialogogues at higher ambient temperatures. The beta adrenergic blocking agent, propranolol, inhibited salivation induced by d-amphetamine less effectively at 30° than at room temperature. In contrast, however, propranolol blocked completely salivation induced by epinephrine and norepinephrine both at 30° and at room temperature. Propranolol was not tested against isoproterenol at elevated ambient temperatures because it did not block this agonist at room temperature.

We have previously reported that salivation induced by dextroamphetamine (7.3 mg base/Kg, i.p.) is completely blocked 4 hr after pretreatment with reserpine (10 mg/Kg, i.p.), but not at 24 hr. However, if the ambient temperature is raised to about 32°C one hr before administration, marked salivation will be induced by d-amphetamine despite the pretreatment with reserpine. Exposure of mice, which have been pretreated with reserpine, to an ambient temperature of 40°C for 30 min will itself induce a salivation response of 7.3, when measured as explained in previous reports, with a possible range of 0 to 18. For comparison, exposure of untreated mice to the same ambient temperature induced a salivation response of 14.3.

Significance to Biomedical Research and the Program of the Institute: This study has increased our understanding of the role of adrenergic salivation in temperature regulation in animals in which sweating is not a major function of heat dissipation. We have also developed a procedure for studying salivation in mice which is well-suited for the demonstration of interactions among autonomic drugs.

Proposed Course of Project: We hope to complete manuscripts which are now in preparation.

Honors and Awards: None

Publications:

Curry, S.H., Davis, J.M., Janowsky, D.S. and Marshall, J.H.L.: Factors affecting chlorpromazine plasma levels in psychiatric patients. Arch. Gen. Psychiat. 22: 209-215, 1970.

Hollister, L.E., Curry, S.H., Derr, J.R. and Kanter, S.L.: Studies of delayed action medication. V. Plasma levels and urinary excretion of four different dosage forms of chlopromazine. Clin. Pharmacol. Ther. 11: 49-59, 1970.

Curry, S.H., Marshall, J.H.L., Davis, J.M. and Janowsky, D.S.: Chlorpromazine plasma levels and effects. Arch. Gen. Psychiat. 22: 289-296, 1970.

Serial No. NHLI-198
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Sodium and Potassium on the Kinetics of Serotonin and Norepinephrine Transport by Rabbit Synaptosomes

Previous Serial Number: None

Principal Investigators: Dr. Donald F. Bogdanski
Dr. Anja H. Tissari

Other Investigators: None

Cooperating Units: Dr. Tissari was an International Fellow

Project Description:

Objectives: To study the dependency of the amine transport mechanism located at the plasma membrane of nerve endings upon the presence of electrolytes. Specifically, to determine whether a specific electrolyte increases or decreases the affinity between the carrier mechanism and organic solute, and whether a facilitative electrolyte forms a ternary complex with carrier and solute.

Methods Employed: Synaptosomes were prepared and incubated in various media according to methods previously published by this laboratory (Tissari et al. Mol. Pharmacol. 5: 593, 1969).

Major Findings: We previously reported that the transport of biogenic amines by nerve endings involved an asymetric distribution of Na^+, K^+ across the plasma membrane. A third asymetry, the relative affinity between carrier and amine was determined by the electrolyte distribution. Thus, Na increases and K decreases the affinity of the carrier for amine. Hence, that affinity is high outside the cell where the concentration of Na^+ is high and K^+ is low, and low inside the cell where the ion concentrations are reversed. This situation may not apply for total electrolytes within the synaptosome, but since the concentration of intracellular Na is high, a local Na gradient may exist by reason of (Na + K)-ATPase activity occurring simultaneously with amine transport (Bogdanski et al., Biochim. Biophys. Acta 211: 521, 1970).

In any event the original premises outlined above were tested in synaptosomes by the application of the principles of kinetics to the problem. The existence of a saturable carrier mechanism was shown by the results of

experiments on the accumulation and transport of 5-HT by synaptosomes incubated in Krebs-HCO_3 medium containing 10 - 1000 ng/ml 5-HT. Accumulation and uptake increased with increasing concentration of 5-HT, a plot of the data being curved at the lower concentrations, then becoming a straight line at concentrations of 5-HT above 150 ng/ml. On the assumption that the straight line portion represented 5-HT entry into the cell by passive diffusion, the amount of 5-HT accumulated or transported by synaptosomes by passive diffusion was calculated and subtracted from the total amount accumulated or transported. The remainder represented that amount that was accumulated or transported by means of the transport process. This carrier was virtually saturated at a concentration of 150 ng/ml. Plotting the reciprocal of transport against the reciprocal of 5-HT concentration produced a straight line having an apparent Km of 71 mM and V_{max} of 1.1 nmoles/mg protein/hr.

Other experiments were designed for representation by double reciprocal plots of the initial rate of accumulation of 5-HT and NE in the presence of various concentrations of Na. The plots suggested that Na acts chiefly by decreasing the apparent Km of 5-HT and NE, although Na slightly increases the V_{max} for 5-HT but not NE. These curves were corrected for that amount of 5-HT that entered the cell passively. The effect of Na on the apparent Km of amines suggests that Na increases the affinity between carrier and amine, but not the maximum velocity of transport.

Plotting the same data in the form of a double reciprocal plot of the initial rate of accumulation of 5-HT and NE against sodium concentration in the presence of various concentrations of amine produced similar but not identical results. Whereas the curves for NE were linear and intersected the y axis at a common point, those plotted for 5-HT were partially linear at best. A linear curve in this plot suggests that amine, Na and carrier cross the membrane as a ternary complex.

High concentrations of potassium antagonized the facilitating effect of Na on transport.

Significance to Biomedical Research and to the Program of the Institute: The nerve endings collectively are vital links in the recognition, integration and organization of the organisms' responses to changes in its environment. The present studies add fundamental information to our understanding of the biochemical functions of nerve endings at the molecular level. Only through such information will it be possible to make rational approach to the control of such responses or to repair these responses when abnormal.

Proposed Course of Project: This aspect of our work on nerve endings is essentially complete.

Honors and Awards: None

Publications: Bogdanski, D. F., Tissari, A. H. and Brodie, B. B.: Mechanism of transport and storage of biogenic amines. III. Effects of sodium and potassium on kinetics of 5-hydrooxytryptamine and norepinephrine transport by rabbit synaptosomes. Biochim. Biophys. Acta 219: 189-199, 1970.

Serial No. NHLI-199
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effect of Divalent Ions on the Kinetics of Release of H^3-NE from Rat Heart Slices

Previous Serial Number: NHLI-261

Principal Investigator: Dr. Donald F. Bogdanski
Dr. Thomas P. Blaszkowski

Other Investigators: None

Cooperating Units: Dr. Blaszkowski is a Hoffmann-LaRoche Fellow.

Project Description:

Objectives: Messages relating to body functions are transmitted from one nerve to another, or from nerve to effector organ, by means of chemical transmitters. These transmitters are released by the arrival of a nerve impulse at the nerve ending. The release is effected by a process which requires the presence of Ca in the extracellular medium, and calcium interacts with Na in this process (Keen and Bogdanski, Am. J. Physiol. 219: 677, 1970). The present report describes detailed studies of the mechanism of release, and especially, the Na-Ca interaction.

Methods Employed: The incubation of rat heart slices for the purpose of investigating the efflux of previously labeled stores of NE have been previously published by this laboratory (Bogdanski and Brodie, J. Pharmacol. Exp. Ther. 165, 181, 1969; Keen and Bogdanski, Am. J. Physiol. 219: 677, 1970).

Major Findings: Previous reports have shown that Ca and Ba increase the rate of efflux of H^3-NE from rat heart slices incubated in Na^+-free media made isotonic with sucrose or choline. The H^3-NE represents an increment over and above the release of radioactivity (80-90% deaminated metabolite) from slices incubated in control Krebs-bicarbonate solution. The reciprocals of the rate constant of release of H^3-NE ($k - k_{Krebs}$), plotted against the reciprocal of Ca or Ba concentration, were linear. These results suggest that the release of H^3-NE by Ca is mediated by a saturable process which obeys Michaelis-Menten kinetics. The process is abolished at 0°C.

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The calcium dependent release is greater in media containing choline or K ions as Na substitutes, than in media in which isotonicity is maintained by Li or sucrose. However, in the presence of small amounts of Na, a slight effect of Ca is observed in the presence of sucrose or Li.

Various concentrations of Na and Ca were added to media containing 143 mM K and the values of $k - k_{Krebs}$ calculated from the results of the experiments were plotted in the usual double reciprocal format. The plots suggest that Na and Ca are non-competitive antagonists.

More detailed work suggested that efflux depended upon the loss of Na from the tissues rather than the entrance of choline into the tissues. Although the loss of Na is accompanied by an increased uptake of Ca (Keen and Bogdanski ibid.), the results of the experiments with sucrose or Li^+ indicate that the presence of Ca^{++} is not an absolute requirement for release in Na deficient media.

The main conclusions to be drawn from this work is that Na is required for storage whether or not Ca is present in the extracellular fluid. Choline and K act as weak Na substitutes that can be antagonized by Ca. Ca, as mentioned earlier, is a non-competitive antagonist of Na. In Na^+-free media, NE is released either because more Ca can enter the cell or because less Na is available to antagonize the NE releasing effect of Ca within the cell or both. Baker et al. showed that Ca and Na move across the plasma membrane in either direction in exchange for one another. Hence, another effect of extracellular Na may be to facilitate the extrusion of intracellular Ca by exchange with Na.

An alternative to the hypothesis described in the above paragraphs makes use of Baker's data showing that the efflux of intracellular Na increases in the presence of extracellular Ca. We have shown that isolated nerve endings (synaptosomes) may contain large amounts of Na (Bogdanski et al., Biochim. Biophys. Acta 211, 521, 1970). The alternative hypothesis suggests that the nerve impulse mobilizes a component of intracellular Na responsible for amine retention. The Na so mobilized exchanges with extracellular Ca to produce a deficiency of Na at some key point required for storage. This idea satisfies the requirement for Ca transmitter release and is in accord with the data showing that Na deficiency alone may correlate with increased rates of efflux.

Significance to Biomedical Research and the Program of the Institute: Ultimately all functions of the body are begun or controlled by the liberation of chemicals which transmit impulses from one nerve to the next step in a chain. The studies reported will aid in the understanding of the molecular processes involved in transmitter release. Such fundamental knowledge is required for a rational approach to the treatment of any dysfunction at this level.

Proposed Course of Project: As time permits, further work will be focused on the role of intracellular electrolytes in the storage and release of nerve transmitters.

Honors and Awards: None

Publications: Blaszkowski, T. P. and Bogdanski, D. F.: Possible role of sodium and calcium ions in retention and physiological release of norepinephrine by adrenergic nerve endings. Biochem. Pharmacol., 1971. In press.

Serial No. NHLI-200
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Effect of Enzyme Induction on Synthesis and Catabolism of Corticosterone in Rats. Analysis by Means of Steady-State Kinetics

Previous Serial Number: NHLI-262

Principal Investigator: Dr. Donald F. Bogdanski
Dr. Thomas P. Blaszkowski

Other Investigators: None

Cooperating Units: Dr. Blaszkowski holds a Hoffmann-LaRoche Fellowship

Project Description:

Objectives: The main purpose of our present studies is to establish techniques suitable for the analysis of synthesis and metabolism of corticosterone in the rat by means of steady-state kinetics. Having done so, it may be feasible to analyze the effects of various drugs, which affect synthesis or catabolism of corticosterone, on plasma levels of steroid. As a corollary, it may be possible to determine whether drugs alter plasma corticosterone levels primarily by changing the rate of synthesis or catabolism or both.

Methods Employed and Major Findings: Corticosterone in rat plasma was estimated by standard procedures.

Two methods used for the estimation of steady-state parameters were compared and found to yield significantly different results. The methods and the results of their use are summarized as follows: The rapid intravenous injection method involves the killing of individual rats at various time intervals after the injection of a constant dose of radioactive tracer. Rate constants of the disappearance (k) of tracer from plasma are estimated from the exponential decline of plasma levels as plotted on semi-logarithmic graph paper. The rate of synthesis is calculated from the steady-state relationship:

Rate of Synthesis, (K) = Rate of metabolism (k) x Steady-state plasma level(C)

The practical disadvantages of this method are manifold. The injection of tracer disrupts the steady-state by causing an outpouring of corticosterone from the adrenals. This effect is slight or absent if the animals are

deeply anesthetized. However, the method requires an accurate estimation of the volume of distribution of drug. This measurement cannot be made accurately when the drug is being metabolized as rapidly as is corticosterone, because the tracer does not come to equilibrium with the multiple components of the whole body pool.

A better method involves the infusion of tracer quantities of radioactive steroid until the steady state is achieved. This requires about 90 min or more of continuous infusion at a constant rate. Upon cessation of the infusion, a biphasic decline in plasma levels occurs, the exponential phase having a half-life (t 1/2) of about 37 min.as compared with 23 min estimated by the single injection method. The difference reflects the equilibration of tracer administered by infusion with the whole body pool. The rate of synthesis is easily calculated, provided that 1. the tracer quantity is too small to influence the whole body pool, 2. the tracer is in equilibrium with the whole body pool, and 3. the rates of infusion and adrenal secretion are constant throughout the experiment. Under these conditions,the plasma concentration of tracer represents a certain small proportion of the concentration of endogenous steroid. The rate of infusion is known, and is related to the rate of adrenal output by the same proportion. We have calculated that the rat adrenals synthesize corticosterone at the rate of about 150 ug/Kg/hr.

Certain drugs, such as phenobarbital, are known to induce liver microsomal NADPH enzymes that can metabolize steroids. Phenobarbital does not reduce plasma levels of corticosterone, however, indicating that the adrenal gland compensates for the increased metabolism of the steroid by increasing its rate of synthesis. Increased metabolizm after enzyme induction is shown in the larger k values calculated by both techniques. Moreover, the steady-state plasma level of tracer, but not endogenous steroid, is lower in the rats subjected to the infusion experiments. The rate of adrenal output of corticosterone in the phenobarbital treated rats is about twice that of controls.

Significance to Biomedical Research and the Program of the Institute: Drugs which alter the synthesis or metabolism of adrenal corticosteroids are potentially harmful to the organism if the drugs produce long-lasting alterations in the plasma levels of these steroids. With phenobarbital, a widely used hypnotic and sedative drug, there is apparently little or no danger because the rat compensates for increased metabolism by increasing the rate of synthesis of corticosterone thereby maintaining constant levels of adrenal corticoid.

Proposed Course of Research: The dynamics of distribution, binding. metabolism and synthesis of steroids in animals will be studied in more detail. Our findings will be applied to drug toxicity where relevant.

Honors and Awards: None

Publications: None

Serial No. NHLI-201
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Comparison of the Effects of K^+-free Media and Ouabain on the Transport of 5-HT and Norepinephrine by Synaptosomes

Previous Serial Number: None

Principal Investigator: Dr. Donald F. Bogdanski
Dr. Anja H. Tissari

Other Investigators: None

Cooperating Units: Dr. Tissari was an International Fellow

Project Description:

Objectives: To aid in the understanding of the biophysical mechanisms involved in the transport of neurohormone by nerve endings. Specifically, to compare the properties of the transport inhibiting effects of two means of inhibiting (Na + K)-ATPase.

Methods Employed: The transport, accumulation and metabolism of biogenic amines were studied by means of techniques previously reported by ourselves.

Major Findings: We have made use of the concepts of transport (defined as the total, carrier mediated movement of amine across the plasma membrane, including that which is subsequently metabolized and diffuses back into the extracellular medium) and accumulation, the ratio of intracellular to extracellular amine. The present data reveal that a K^+-free medium and ouabain both inhibit transport to a greater extent than accumulation indicating that the transport process was more severely affected than previously realized.

The requirement of Na gradient as the energy source for active transport and accumulation was tested in K^+-free media and compared with that in media containing ouabain. Ouabain and K^+-free media inhibit (Na + K)-ATPase almost immediately whereas both inhibit 5-HT transport after a measurable time lag, the development of inhibition being dependent upon the presence of Na^+ in the extracellular fluid (Tissari et al., Mol. Pharmacol. 5: 593, 1969; Bogdanski et al., Biochim. Biophys. Acta 211: 521, 1970). Hence, it was proposed that the inhibition of transport by (Na + K)-ATPase inhibitors was the result of a secondary, time dependent process involving extracellular Na^+. The abolition of transport was thought to depend upon the abolition of the Na^+ gradient across the cell membrane. The report of Tissari et al. showed that NE,

unlike 5-HT, was not transported in the presence of ouabain after a Na gradient was re-established by the addition of Na^+ to the medium.

The present data also show that the inhibition of transport of NE in K^+-free medium occurs after a time lag as previously reported for 5-HT. The present experiments indicate that the inhibition of $(Na^+ + K^+)$-ATPase by K^+-free media inhibits the transport of NE. The transport can be re-started by adding Na^+ to the medium, thus re-establishing a Na gradient. Hence, the failure of synaptosomes to transport NE in the presence of ouabain must be due to the presence of ouabain per se, and not due to the inhibition of (Na + K)-ATPase.

The significance of Na concentration in the temporal development of the inhibitory effect of K-free media was also shown by adding 5-HT to a suspension of synaptosomes in media containing either 50 or 150 mM Na, after a 10 min preliminary incubation. The medium containing a high concentration of Na failed to transport 5-HT whereas the medium containing a low concentration of Na transported 5-HT to the extent of 42% of control. Thus, the delay in the development of the inhibitory effect of K-free media as the transport process is dependent upon the concentration of Na in the medium.

The same observation was made for ouabain. At a concentration of 10^{-4}M in a medium containing 50 mM Na, ouabain blocked accumulation and transport of 5-HT by 41% and 63%, respectively after 10 min preliminary incubation, ouabain in a medium containing 150 mM Na blocked accumulation and transport of 5-HT by 74% and 95% respectively.

In this and a number of previous reports, a general similarity was noted in the characteristics of transport block by ouabain and by K^+-free media. It was thought that ouabain might produce a functional deficiency of K at some point essential for transport. High concentrations of K are known to antagonize the inhibitory effect of ouabain on (Na + K)-ATPase. At concentration of 75 mM (itself inhibitory) potassium decreased the inhibitory effect of 10^{-4}M ouabain upon accumulation and transport of 5-HT from 74% and 95% to 45% and 69%, respectively.

In conclusion, ouabain blocks transport of biogenic amines by producing functional deficiency of K^+ at some key point required for transport. Moreover, inhibition of amine transport by ouabain and K^+-free media resemble their inhibition of (Na + K)-ATPase. The key point in question is whether ouabain and K^+-free media inhibit transport directly or indirectly by inhibiting $(Na^+ + K^+)$-ATPase or another enzyme having very similar properties. All our assumptions relating to the delayed inhibition of amine transport, contrasting with the rapid inhibition of (Na + K)-ATPase, are supported by the present data.

Significance to Biomedical Research and the Program of the Institute: The various responses of the organism to changes in the environment in order to maintain homeostasis, are integrated, controlled and mediated by means of nerve endings. The experiments reported expand our knowledge of the physi-

ology and biochemistry of nerve endings at the molecular level. Such basic knowledge is required before rational approaches to control or therapy can be undertaken.

Proposed Course of Project: This phase of our work on the nerve ending is terminated.

Honors and Awards: None

Publications: Tissari, A. H. and Bogdanski, D. F.: Biogenic amine transport VI. Comparison of effects of ouabain and K^+ deficiency on the transport of 5-hydroxytryptamine and norepinephrine by synaptosomes. Pharmacology, 197. In press.

Serial No. NHLI-202
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of Electrolytes on the Accumulation and Metabolism of Biogenic Amines by Subcellular Fractions of Rat Brain

Previous Serial Number: None

Principal Investigators: Dr. Donald F. Bogdanski
Dr. Anja H. Tissari

Other Investigators: None

Cooperating Units: Dr. Tissari was an International Fellow

Project Description:

Objectives: To compare active transport and metabolism of biogenic amines by various subcellular fractions of rat brain in order to establish the site of metabolism of amines by synaptosome fractions and to compare the relative purity of various fractions.

Methods Employed: The techniques used for the isolation and incubation of synaptosomes have been previously reported. Other subcellular fractions were isolated simultaneously.

Major Findings: Whole brains of rats and rabbits were used in these studies and divided into the following fractions: supernatant, myelin and debris, membrane fragments and small synaptosomes, synaptosomes, mitochondria and large synaptosomes (mitochondrial fraction) and red blood cells and large fragments. These fractions were studied for their monoamine oxidase (MAO) content using serotonin (5-HT) as substrate. About 15% of the MAO was contained in the supernatant fraction, whereas peaks of activity occurred in all other fractions except that containing the red-blood cells and tissue fragments. Metabolism in the synaptosomal fraction was mediated chiefly by MAO within intact synaptosomes, which are to be considered as isolated cells having an intact plasma membrane. Metabolism in the mitochondrial fraction was mediated chiefly by free mitochondria as well as intact synaptosomes. In order to compare the relative contribution of intact synaptosomes and free mitochondria to total metabolism, we made use of previous data showing that exogenous 5-HT must be carrier transported into the synaptosome before metabolism can occur, metabolism and accumulation being inhibited by ouabain or by Na^+ and/or K^+-free media which curtail or block transport.

In the present investigations, metabolism of 5-HT by synaptosomes incubated in Na^+-free medium was inhibited by 3 times as much as metabolism by synaptosomes in the fraction containing free mitochondria and large synaptosomes. Moreover in the control medium (Krebs-HCO_3) the synaptosome fraction accumulated much more 5-HT than did the mitochondrial fraction. The large amount of radioactivity present in the mitochondrial fraction was present chiefly as deaminated metabolite. These results show that amines diffuse freely to the site of metabolism in mitochondria, and do not require Na^+ mediated transport for metabolism. By contrast, amines must be transported into synaptosomes before undergoing metabolism. In accord with observations by means of the electron microscope, the various fractions are relatively pure biochemically but are not absolutely pure.

Significance to Biomedical Research and the Program of the Institute: These studies add fundamental knowledge of the biochemical physiology of nerve endings and mitochondria. The nerve ending is, of course, essential to life and our awareness and response to the environment.

Proposed Course of Project: This phase of our studies on nerve endings is ended.

Honors and Awards: None

Publications: The above material was published as previously unpublished data in a review of our work:
Tissari, A. H. and Bogdanski, D. F.: Effects of inorganic electrolytes on the membrane transport and metabolism of serotonin and norepinephrine by synaptosomes. International Symposium on the Histochemistry of the Synapse, Ed. O. Eränko, Helsinki, Finland, 1970. In press.

Serial No. NHLI- 203

1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Relationship between Potassium and the Sodium Requirement for Transport and Storage of 5-Hydroxytryptamine and Norepinephrine by Synaptosomes

Previous Serial Number:

Principal Investigator: Dr. Donald F. Bogdanski
Dr. Anja H. Tissari
Dr. Thomas P. Blaszkowski

Other Investigators: None

Cooperating Units: Dr. Tissari was an International Fellow
Dr. Blaszkowski is a Hoffmann-La Roche Fellow

Project Description:

Objectives: To study the ionic requirements of the transport mechanism, located at the plasma membrane, by which exogenous amines are transported into the cell. This mechanism is presumably the same as that which recaptures nerve transmitter released by the nerve impulse. The present data enlarge upon the model transport system we have reported, (Bogdanski and Brodie, J. Pharmacol. Exp. Ther. 165:165-181, 1969).

Methods Employed: The methods used for the isolation and incubation of synaptosomes have been previously reported by this laboratory (Tissari et al., Mol. Pharmacol. 5: 593, 1969).

Major Findings: For this report we have made use of the terms accumulation and transport. Accumulation is ratio of internal to external amine concentration. Transport is the total amount of amine transported into the cell by means of the carrier mechanism, including that which has been metabolized and subsequently lost from the cell. In a medium containing 6 mM K, the initial rate of accumulation and transport of serotonin and norepinephrine increase with increasing concentrations of Na in the medium, until a maximum rate is achieved (Bogdanski et al., Biochim. Biophys. Acta 211:521, 1970). However, in similar but K-free media, initial rates of accumulation and transport of both amines increase up to a maximum at about 50-75 mM Na,

then decreases as more Na^+ is added to the media. Thus, the requirement for Na^+ for transport is modified by the absence of K^+.

The development of inhibition of accumulation and transport of serotonin and norepinephrine by K^+-free media is time dependent, suggesting that K^+-free media act indirectly, since the deficiency of K is instantaneous. Moreover, the relative inhibitory effect of K is greater in media containing 150 mM Na than in media containing 50 mM Na. A possible explanation for the inhibition is that the developing inhibition of transport is the result of the abolition of Na^+ gradients across the membrane. The equalization of ion concentrations is caused by the inhibition of the Na-pump. Measurements of intracellular electrolytes revealed that freshly prepared synaptosomes contain relatively low quantities of electrolytes, the Na and K concentrations increasing during incubation. The final concentration of Na^+ in K^+-free media depends upon the concentration of Na in the media. In the presence of 10^{-3}M ouabain, or in K^+-free media, the intracellular concentration of K is lower than that of controls but the concentration of Na, already high, is not increased. This result suggests that the electrolyte pump can maintain a gradient of K^+ across the membrane, if not Na^+.

It is concluded that transport and accumulation of amines by freshly prepared synaptosomes in K^+-free media becomes inhibited as the level of intracellular Na rises, the amount of inhibition depending upon the concentration of intracellular Na^+. However, transport can occur when intracellular Na is high, approaching or exceeding the extracellular concentration. Under these conditions, the transport of amines will take place only if electrolyte transport occurs simultaneously. This led us to speculate that the transport of amines is energized by the existence of local electrolyte gradients at the membrane-storage vesicle junction or that the $(Na^+ + K^+)$-ATPase may remove electrolyte from the carrier.

Transport of serotonin and norepinephrine is not only inhibited by K-free media, but by high concentrations of K as well. However, transport at 75 mM K was greater than in K^+-free media, suggesting that K fulfills some requirement for the transport process. Hence the facilitative and inhibiting functions of K may be exerted at different parts of the transport process.

The efflux of stored NE from synaptosomes is increased slightly in K^+-free media, but much less than in Na^+-free media.

Significance to Biomedical Research and to the Program of the Institute:
The nerve endings collectively are vital links in the recognition, integration and organization of the organisms' responses to changes in its environment. The present studies add fundamental information to our understanding of the biochemical functions of nerve endings at the molecular level. Only through such information will it be possible to make rational approach to the control of such responses or to repair these responses when abnormal.

Proposed Course of Project: As time permits, the significance of the relationship of transport to the intracellular electrolyte concentration, in terms of our model transport mechanism, will continue to be studied.

Honors and Awards: None

Publication:

Bogdanski, D.F., Blaszkowski, T.P. and Tissari, A.H.: Mechanisms of biogenic amine transport and storage. IV. Relationship between K^+ and the Na^+ requirement for transport and storage of 5-hydroxytryptamine and norepinephrine in synaptosomes. Biochim. Biophys. Acta 211: 521-532, 1970.

Leitz, F.H. and Stefano, F.J.E.: Effect of ouabain and desipramine on the uptake and storage of norepinephrine and metaraminol. Europ. J. Pharmacol. 11: 278-285, 1970.

Leitz, F.H.: Mechanisms by which amphetamine and desipramine inhibit the metaraminol-induced release of norepinephrine from sympathetic nerve endings in rat heart. J. Pharmacol. Exp. Ther. 173: 152-157, 1970.

Leitz, F.H. and Stefano, F.J.E.: Desipramine-induced release of norepinephrine from heart. Biochem. Pharmacol. 19: 1797-1801, 1970.

Serial No. NHLI-204
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Enhancement of Motor Activity in Rats following Withdrawal of Chronic Treatment with Alpha-Methyltyrosine.

Previous Serial Number: None

Principal Investigator: Dr. Jorge Perez-Cruet

Other Investigators: Dr. William E. Bunney
Dr. Dennis Murphy

Cooperating Units: Dr. Perez-Cruet is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.
Dr. William E. Bunney and Dr. Dennis Murphy are with the Section on Psychiatry, Laboratory of Clinical Sciences, NIMH.

Project Description:

Objectives: The present study was designed to determine the mechanisms involved in the enhancement of motor activity which has been observed in humans following chronic treatment with Alpha-methyl tyrosine (Engelman et al., J. Clin. Invest. 47: 577, 1968). This enhancement of motor activity has also been observed in depressed patients treated chronically with alpha-methyltyrosine (Brodie, K. H. et al., Clin. Pharmacol. Ther. 12: 218, 1971).

Methods Employed: Two groups of male rats weighing 200 mg were studied for periods ranging from one week to two months. One control group received saline injections (1 ml/rat) and another group received alpha-methyl tyrosine methyl ester injections (100 mg/kg, i.p.) for 5 days. Motility of the rats was measured over a 24 hr period for periods ranging from one week to two months with an electronic motility meters. Analysis of monoamines were performed at days 1, 3 and 5 after withdrawal of alpha-methyl tyrosine treatment. Both dopamine and homovanillic acid were measured in controls and treated animals. Tyrosine hydroxylase activity in brain was measured with the method of Nagatsu (Nagatsu et al., Anal. Biochem. 9: 122, 1964).

Major Findings: The results showed a significant increase in motor activity between days 2 through 7 following withdrawal of 5 days-treatment with alpha-methyltyrosine. This enhanced motor activity was accompanied by a 14% increase in the steady state levels of brain dopamine and a 140% increase in the steady state levels of brain homovanillic acid. Tyrosine hydroxylase activity was increased by about 19%. These preliminary findings

suggest some significant alterations in the metabolism of dopamine in rats following treatment with alpha-methyltyrosine. The data suggest an activation of dopaminergic neurons possibly due to an induction of tyrosine hydroxylase.

Significance to Biomedical Research and the Program of the Institute: This is the first evidence that dopamine metabolism may be altered after withdrawal of alpha-methyltyrosine.

Proposed Course of Project: Presently we are studying if the turnover of dopamine is changed after withdrawing treatment with alpha-methyl-tyrosine. ^{14}C-tyrosine as a precursor of dopamine is being used to measure the turnover of dopamine. The role of other monoamines, in particular serotonin, is also under study.

Honors and Awards: None

Publications: None

Serial No. NHLI-205
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evidence for a Balance in the Basal Ganglia between Cholinergic and Dopaminergic Activity

Previous Serial Number: None

Principal Investigator: Dr. Jorge Perez-Cruet
Dr. Gian L. Gessa

Other Investigators: Dr. Alessandro Tagliamonte
Dr. Paola Tagliamonte

Cooperating Units: Dr. Paola Tagliamonte held a Geigy Fellowship
Dr. Perez-Cruet is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: The effect of centrally acting cholinergic and anticholinergic drugs on dopamine metabolism in the basal ganglia or rabbits and rats was studied. These studies arose from our previous observations that cholinergic drugs such as oxotremorine, which is used to produce an experimental model for Parkinson's disease in animals, increases the levels of homovanillic acid (one of the main metabolites of dopamine) in the basal ganglia. On the basis of the previous laboratory findings (Corrodi, Life Sci. 6: 2557, 1967) it is evident that a balance between dopaminergic and cholinergic activity is important in extrapyramidal functions. We have hypothesized that the balance between these two systems may play a role in maintaining the physiological regulation of some extrapyramidal functions. Therefore, an increase in dopaminergic activity due to stimulation of cholinergic activity can be an intermediate step in the physiological activation of extrapyramidal functions.

Methods Employed: Rabbits and rats were used to evaluate the effects of cholinergic and anticholinergic drugs on the levels of dopamine and homovanillic acid (HVA) in the basal ganglia. Cholinergic drugs: oxotremorine was injected intraperitoneally (i.p.) in doses ranging from 0.5 mg/kg to 1.0 mg/kg and eserine in doses of 0.2 mg/kg. Another group of animals was treated with an anticholinergic drug atropine (5 mg/kg, i.p.).

The effects of cholinergic and anticholinergic drugs on dopamine synthesis rate was also measured using the method of Neff et al. (J. Pharmacol. Exp. Ther. 160: 48, 1968) by blocking the transport of HVA with probenecid and measuring the rate of accumulation of HVA in the basal ganglia. Turnover

of dopamine was also measured after i.v. pulsed injection of labeled ^{3}H-tyrosine with the method of Sedvall et al. (J. Pharmacol. Exp. Ther. 159: 274, 1968) by measuring the conversion of ^{3}H-tyrosine to ^{3}H-dopamine.

Major Findings: In rabbits, oxotremorine (0.5 mg/kg, i.p.) increased the levels of HVA by about 26% ($p < 0.01$) and increased steady state level of dopamine by about 22% ($p < 0.001$). In rabbits eserine (0.2 mg/kg, i.p.) decreased the levels of dopamine in the basal ganglia by 21% ($p < 0.01$). Atropine decreased the levels of dopamine by about 8% ($p > 0.1$). In rats, eserine (0.2 mg/kg, i.p.) decreased dopamine levels by 29% ($p < 0.01$) and increased HVA concentrations by 18% ($p < 0.05$). Oxotremorine (0.5 mg/kg) decreased dopamine levels by 39% ($p < 0.01$) and increased HVA by 29% ($p < 0.02$). Oxotremorine and eserine significantly increased the accumulation of HVA in the basal ganglia after probenecid (200 mg/kg, i.p.). Oxotremorine significantly increased the conversion of ^{3}H-tyrosine to ^{3}H-dopamine. In rabbits, the synthesis rate of dopamine in the basal ganglia in control was 0.16 µg/g/hr; atropine: 0.14 µg/g/hr (-10% decrease); eserine 0.17 µg/g/hr (11% increase); and oxotremorine: 0.26 µg/g/hr (66% increase). In rats, the synthesis rate of dopamine in basal ganglia was 0.05 µg/g/hr; atropine: 0.06 µg/g/hr (20% increase); eserine: 0.098 µg/g/hr (96% increase) and oxotremorine: 0.15 µg/g/hr (194% increase). These results indicate that the turnover of dopamine is increased by cholinergic stimulation.

Significance to Biomedical Research and the Program of the Institute: This is the first study showing a balance between dopaminergic and cholinergic activity in the basal ganglia of rabbits and rats.

Proposed Course of Project: Measurement of turnover of dopamine will be done with other cholinergic and anticholinergic drugs to determine to what extent different cholinergic drugs stimulate dopamine turnover.

Honors and Awards: None

Publications:

Forn, J., Gessa, G.L., Krishna, G. and Brodie, B.B.: Increased lipolytic response to norepinephrine in isolated brown fat cells after sympathetic denervation. Life Sci. 9: 429-435, 1970.

Gessa, G.L., Krishna, G., Forn, J., Tagliamonte, A. and Brodie, B.B.: Bheavioral and vegetative effects produced by dibutyryl cyclic AMP injected into different areas of the brain. In Greengard, P. and Costa E. (eds.): Role of Cyclic AMP in Cell Function. Advances in Biochemical Psychopharmacology, Vol. 3, New York, Raven Press, 1970, pp. 371-381.

Sjoerdsma, A., Lovenberg, W., Engelman, K., Carpenter, W.T., Jr., Wyatt, R.J. and Gessa, G.L.: Symposium on Serotonin Now: Clinical Implications of Inhibiting its Synthesis with para Chlorophenylalanine. Ann. Intern. Med. 73: 607-629, 1970.

Serial No. NHLI -206
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Cerebral Serotonin Metabolism and Tolerance to Selective REM-Sleep Deprivation in Normal and Hypophysectomized Rats
Previous Serial No.	None
Principal Investigators:	Dr. H. Cramer Dr. J. Perez-Cruet Dr. A. Tagliamonte Dr. P. Tagliamonte Dr. G.L. Gessa
Other Investigators:	None
Cooperatung Units:	Dr. Perez-Cruet is a Research Associate in the Pharmacology-Toxicology Program, NIGMS Dr. P. Tagliamonte holds a Geigy Fellowship

Project Description:

Objectives: REM-sleep deprivation has been reported to increase brain serotonin turnover in rats (F. Hery et al.: Brain Research 21: 319, 1970). Experiments were carried out to clarify whether this effect was the consequence of pituitary activation by stress.

Methods Employed: Intact and hypophysectomized male rats were REM-sleep deprived according to Pujol et al. (Pujol et al.: Science 159: 112, 1968).

Major Findings: In normal rats REM-sleep deprivation for 3,5 and 7 days produced respectively: no change, 20 and 35% increase in brain 5-hydroxy-indole acetic acid (5-HIAA). Brain serotonin and tryptophan levels were increased by about 25% after 5 or more days of REM sleep deprivation.

After 7 days of REM-sleep deprivation, plasma corticosterone fell to about 60% of the normal level.

Hypophysectomized rats survived for only up to 4 days of REM-sleep deprivation. In these animals,3 days of REM-sleep deprivation increased the brain 5-HIAA by 100%, the brain tryptophan by 50% and the brain serotonin levels by 20%.

The results suggest that the changes of serotonin metabolism induced by REM-sleep deprivation are not due to pituitary stimulation; on the contrary, they seem to be secondary to corticotrophin deficiency.

Significance to Biomedical and the Program of the Institute: These investigations provide the first evidence that changes in serotonin metabolism due to REM-sleep deprivation are not secondary to pituitary stimulation.

Proposed Course of Project: We plan to continue these studies in adrenalectomized rats to determine the role of adrenals in cerebral serotonin metabolism and tolerance to selective REM-sleep deprivation. Studies of corticosterone turnover in hypophysectomized rats subjected to sleep deprivation are also planned to determine if the corticotropin deficiency changes the turnover of corticosterone in REM-sleep deprived animals.

Honors and Awards: None

Publications: None

Serial No. NHLI-207
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Alterations in Brain Monoamines and their Amino Acid Precursors in Chronically Jaundiced (Gunn) Rats

Previous Serial Number: None

Principal Investigators: Dr. Jorge Perez-Cruet
Dr. Anthony M. Guarino

Other Investigators: None

Cooperating Unit: Dr. Guarino is a member of the Laboratory of Chemical Pharmacology, National Cancer Institute

Project Description:

Objectives: The present study was designed to determine the effects of chronic hyperbilirubinemia on brain levels of monoamines; serotonin, dopamine and norepinephrine; and their amino acid precursors: tryptophan and tyrosine. These preliminary studies were performed in Gunn rats, which are known to have a genetic deficiency of uridine diphosphateglucuronyl transferase in the liver microsomes (Axelrod et al., Nature 180:1426, 1957). Gunn rats have been used as an experimental model to study the toxic effects of bilirubin in the brain (Schutta, H.S., et al., J. Pediatrics 75:1070, 1969).

Methods Employed: Three groups of rats were used: 1) 6 control male Wistar rats, 2) 6 homozygous male Gunn rats and 3) 6 heterozygous male Gunn rats.

Separation of brain monoamines and amino acids was done by ion-exchange chromatography and assays were done by standard spectrofluorometric methods. Total serum bilirubin was measured both spectrofluorometrically (Roth M., Clinica Chimica Acta 17:487, 1967) and colorimetrically (Nosslin, B., Scand. J. Clin. Lab. Invest. 12, Supp. 49:1, 1960). Liver microsomal bilirubin uridine diphosphate-glucuronyl transferase activity was determined using phenolphthalein as a substrate.

Major Findings: All homozygous Gunn rats showed a deficiency of bilirubin uridine diphosphate-glucuronyl transferase in liver microsomes and high levels of total serum bilirubin (controls: 0.3 to 1.26 mg/100), homozygous Gunn rats: 17.6 mg/100 ml; a significant change in brain tyrosine (controls: 16.1 µg/g;

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homozygous Gunn rats 7.8 µg/g), and an increase in 5-HIAA (controls: 0.91 µg/g; homozygous 1.15 µg/g), but no significant change in the levels of serotonin, tryptophan or dopamine. Heterozygous Gunn rats showed no change in tyrosine, dopamine or norepinephrine levels, but they had higher levels of serotonin (controls 0.68 g/g; heterozygous Gunn rats: 0.80 g/g) and 5-HIAA (controls 0.91 g/g: heterozygous Gunn rats: 1.29 g/g); two heterozygous Gunn rats showed a deficiency of uridine diphosphate-glucuronyl transferase and jaundice.

These preliminary findings suggest that in icteric Gunn rats there are alterations in the metabolism of catecholamines. The increase in 5-HIAA and serotonin suggest possible alterations in serotonin metabolism. Changes in tyrosine levels in brain probably reflect changes in structural protein in nerve cells affected by chronic hyperbilirubinemia.

Significance to Biomedical Research and the Program of the Institute: These studies provide the first evidence that some brain amino acids and monoamines are altered in chronic hyperbilirubinemia in Gunn rats. The study is the first in a series to determine the mechanisms of bilirubin toxicity in the brain.

Proposed Course of Project: We plan to determine whether the turnover of norepinephrine, dopamine or serotonin are changed using ^{3}H-tyrosine and ^{14}C-tryptophan as precursors. The levels of bilirubin in tissues, specially brain tissue will be determined to investigate the possible interrelations between brain levels and alterations in brain monoamine metabolism.

Honors and Awards: None

Publications: None

Serial No. NHLI-208
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Dynamic Aspects of Hormonal Control of the Formation of Cyclic AMP in Human and Rabbit Platelets

Previous Serial Number: NHLI-216

Principal Investigators: Dr. James Harwood
Dr. Jay Moskowitz
Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: Dr. Moskowitz is Research Associate in the Pharmacology-Toxicology Program, NIGMS.

Project Description:

Objectives: Cyclic AMP has now been accepted as an intracellular mediator of the response of a variety of hormones. This conclusion has been drawn from numerous studies in which changes in the level of the cyclic nucleotide have been correlated with changes produced by the hormone, e.g. epinephrine induces phosphorylase activation in cardiac and skeletal muscle, and various hormones stimulate lipolysis in fat cells. In order to characterize the role of cyclic AMP, it is necessary to determine the factors which govern the intracellular level of cyclic AMP. Platelets offer a suitable single cell system for such studies. In an earlier part of this project, it was shown that prostaglandin E_1 (PGE_1) was potent in stimulating both the intracellular accumulation of cyclic AMP in intact platelets and the adenyl cyclase activity in platelet homogenates. In intact platelets, norepinephrine alone has no stimulant action but inhibited the action of PGE_1. The effect of norepinephrine was mediated by alpha receptors, since the effect was inhibited by phentolamine, an alpha adrenergic blocking drug, but not by propranalol, a beta adrenergic blocking drug.

The present studies were undertaken to investigate the dynamic aspects of the formation of cyclic AMP in intact platelets. The ^{3}H-adenine method has been used to monitor changes in the intracellular level of cyclic AMP. Platelets were preincubated with ^{3}H-adenine which is taken up by the cells and converted to ^{3}H-ATP which serves as a substrate for adenyl cyclase. Since the validity of the ^{3}H-adenine method as an indication of the behaviour of endogenous cyclic-AMP levels has been questioned, total platelet cyclic AMP levels have also been measured. These measurements enabled

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comparison of drug-induced changes in total and ^{3}H-cyclic AMP levels.

Methods Employed: Human platelets were prepared from fresh human blood or human platelet concentrates, both supplied by the NIH blood bank. Rabbit platelets were prepared from anesthesized rabbits. Platelets were preincubated with ^{3}H-adenine, washed, resuspended, and added to polycarbonate vials containing the drugs. ^{3}H-cyclic AMP was isolated and counted by the method of Krishna. Total cyclic AMP levels were measured by a modification of the protein kinase binding assay of Gilman.

Major Findings: 1. PGE_1 caused a rapid increase in the level of ^{3}H-cyclic AMP in both species, reaching a peak within seconds. In human platelets the level remained constant, but in those of rabbits it declined by 2/3 in 10 min. 2. The stimulant action of PGE_1 was observed in the absence of theophylline. In the presence of 2×10^{-3}M theophylline, the peak level of cyclic AMP was doubled. 3. The formation of cyclic AMP was temperature dependent. At 20°C, the peak cyclic AMP level occurred after the same time interval as at 37°C but was of smaller magnitude. These effects of temperature were observed in the absence or presence of theophylline. 4. Previously, it was shown that norepinephrine inhibited the stimulant action of PGE_1. It has now been shown that when cyclic AMP levels are elevated by preincubation of blood platelets with PGE_1 for 1 min, addition of norepinephrine caused a rapid dose-dependent decrease in cyclic AMP levels. This effect was not inhibited by theophylline. 5. Relative changes in ^{3}H-cyclic AMP (^{3}H-adenine method) and total cyclic AMP (protein kinase binding method) corresponded closely.

Significance to Biomedical Research and to the Program of the Institute: If cyclic AMP is the mediator of hormone action, then the factors governing the intracellular level of the nucleotide are critical to the expression of hormonal action. The present findings indicate the following: 1. Cyclic AMP formation in response to hormones (PGE_1) is rapidly stimulated, and the increased concentration of the cyclic nucleotide may act as a pulse or a signal for subsequent biochemical events. From these considerations, studies on the adenyl cyclase in cell-free systems may bear little relation to intracellular events. 2. Human and rabbit platelets behave slightly differently. Although in both species peak levels of cyclic AMP are rapidly achieved, in rabbit the levels decline over a 10 min period, in spite of the presence of theophylline. 3. Net cyclic AMP levels are the result of synthesis and degradation. If cyclic AMP is undergoing rapid turnover during hormonal stimulation, the net conversion gives no indication of the rate at which ATP is being converted to cyclic AMP at the "steady-state" level. 4. The effects of theophylline cannot be adequately explained in terms of phosphodiesterase inhibition.

There are several indications that the ability of platelets to aggregate is related to the level of cyclic AMP in platelets. Agents which promote aggregation decrease cyclic AMP levels and agents which inhibit aggregation cause an increase in the level of the cyclic nucleotide. Whether there is a

causal relationship between cyclic AMP level and aggregation has yet to be established. In this regard, a cyclic AMP dependent protein kinase has been found in platelets which will promote phosphorylation of proteins. It is possible that transfer of phosphate groups could be important in platelet aggregation.

Proposed Course of Project: 1. To study the effect of drugs reported to influence platelet aggregation on platelet cyclic AMP levels, e.g. dipyridamole and analogues. 2. To study the hormonal specificity of the platelet adenyl cyclase system by studying the effects of insulin, ACTH and glucagon. 3. To investigate the ionic requirements for hormonal activation of platelet adenyl cyclase. 4. To purify plasma membranes from platelets and study PGE_1 binding and catalytic activity of the PGE_1 sensitive adenyl cyclase.

Honors and Awards: None

Publications: Moskowitz, J., Harwood, J. P., Reid, W. D. and Krishna, G.: The interaction of norepinephrine and prostaglandin E_1 on the adenyl cyclase system of human and rabbit blood platelets. Biochim. Biophys. Acta 230: 279-285, 1971.

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Serial No. NHLI- 209
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Biliary Excretion of Bromobenzene Metabolites

Previous Serial Number: None

Principal Investigators: Dr. I. Glenn Sipes
Dr. Philippe Gigon
Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: To determine whether metabolites of bromobenzene are actively secreted from the liver into the bile and whether agents that alter the liver microsomal drug metabolizing enzymes also affect the rate of secretion of these metabolites.

Methods Employed: Male rats, 200-250 gm (Hormone Assay) were anesthetized with pentobarbital (50 mg/Kg, i.p.) and barbital (100 mg/Kg, i.p.). The bile duct was cannulated and bile collected into a pre-weighed counting vial. Rats were pretreated with phenobarbital (80 mg/Kg, i.p.) for 3 days prior to surgery. SKF 525-A (80 mg/Kg, i.p.) was administered 2 hr prior to surgery. After approximately a 1/2 hr stabilization period, subtoxic doses of ^{14}C-bromobenzene (3.5 to 4.5 mg/200 gm; 25 to 50 μCi) was administered i.v. At 1/2 hr intervals the bile and an aliquot of plasma were collected. After 3 hr, the final bile and plasma samples were obtained, the urine collected and the animal sacrificed. Total radioactivity for each 1/2 hr sample was determined, as well as the total radioactivity for an aliquot of urine. Aliquots (10 μl) of bile and urine were subjected to paper chromatography for separation of various metabolites.

Major Findings: Biliary excretion of bromobenzene accounts for a major percentage of the administered dose. For example, in 3 hr an average of 56% of the administered dose can be accounted for in the bile. An additional 25% of the administered dose is present in the urine. Phenobarbital pretreatment results in an enhanced excretion of bromobenzene metabolites - 50% of the administered dose being excreted in the bile in the first hr, as compared with 28% in the first hr for controls. However, the cumulative total excretion of bromobenzene metabolites in 3 hr is only slightly higher in phenobarbital pretreated animals (65%) as compared to controls (56%).

Preliminary experiments indicate that SKF 525-A inhibits the excretion of biliary metabolites of bromobenzene at least in the first hr - only 18% being excreted.

Bromobenzene rapidly disappears from plasma. At the end of the first 30 min less than 5% of the injected dose is present in the total plasma of the rat as bromobenzene.

Analysis of paper chromatograms indicate that there are several bromobenzene metabolites present in the bile. In most cases there are 4 peaks; the major metabolites have a Rf value that corresponds to the bromobenzene-GSH conjugate. There are two major metabolite peaks present in the urine. One peak corresponds to mercapturic acid of bromobenzene, but the Rf is slightly less (0.84 compared to 0.92). This metabolite probably represents the premercapturic acid. The other urinary peak disappears after 12-16 hr incubation with glucuronidase indicating a glucuronic acid conjugating phenolic metabolites of bromobenzene. There appeared to be no change in the pattern of metabolites in the bile after glucuronidase treatment, indicating that no glucuronide metabolites of bromobenzene are excreted into the bile.

Significance to Biomedical Research and the Program of the Institute: The interesting finding that glutathione conjugates of bromobenzene are excreted into the bile, it may be possible to study the formation of GSH conjugates of certain drugs like diphenylhydanloin in vivo through an active intermediate which then may form a GSH conjugate and may be excreted into the bile. The analysis of urinary metabolites in these cases may be misleading in that they may undergo further metabolism and obscure the formation of GSH conjugate in the liver. Moreover, this finding supports the hypothesis that GSH conjugate is the precursor to mercapturic acid of bromobenzene which is normally excreted in the urine. It is possible that small intestine plays an important role in the metabolism of GSH conjugate to a cysteine conjugate by the glutathionase present in the small intestine. This conjugate may then be converted to mercapturic and by acetylation of cysteine moiety in the liver which is then excreted into the urine.

Proposed Course of Project: Since several metabolites are found in the bile, these metabolites should be identified. Also, other compounds that are metabolized similar to bromobenzene will be investigated to determine the importance of biliary excretion of metabolites and their capacities to produce tissue lesions.

Honors and Awards: None

Publications: None

Serial No. NHLI-210
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Effects of Phenobarbital and 3-Methylcholanthrene Pretreatment on the *in vitro* Metabolism of Bromobenzene in 3 Species

Previous Serial Number: None

Principal Investigator: Dr. I. Glenn Sipes
Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: It is known that animal species do not metabolize foreign compounds at the same rate or by the same pathway. Moreover certain agents that alter the microsomal enzyme system, do not produce the same effect in all animal species. The objective of this project was to study the metabolism of bromobenzene in rats, rabbits and mice after pretreatment with phenobarbital and 3-methylcholanthrene.

Methods Employed: Rats were pretreated with drugs according to the following schedule: a) Phenobarbital - rats and mice, 80 mg/Kg, i.p. for 3 days prior to isolation of microsomes; rabbits 60 mg/Kg, i.p. for 3 days. b) 3-Methylcholanthrene (3-MC) - all species, 20 mg/Kg, i.p. in sesame oil every 12 hr for 3 doses. Microsomes were isolated 24 hr after last dose. ^{14}C-bromobenzene was incubated with the microsomes and the GSH-bromobenzene conjugate isolated as described in the preceding report.

Major Findings: Microsomes from all species studies (rat, rabbit and mouse) metabolize bromobenzene. It appears that liver microsomes from the mouse and rabbit have a higher basal activity for bromobenzene metabolism than microsomes from the rat. Phenobarbital greatly increases the capacity for bromobenzene metabolism by microsomes from all species. This is particularly so for rat liver microsomes in which the formation of bromobenzene-GSH conjugate is 20 times greater after phenobarbital induction. It is interesting that 3-methylcholanthrene enhances bromobenzene metabolism in both the rat and mouse, but produces an inhibitory effect on microsomes from the rabbit. These data are summarized in the following table:

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EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE PRETREATMENT ON THE METABOLISM OF ^{14}C-BROMOBENZENE BY LIVER MICROSOMES

	picomoles/min/mg microsomal protein ± S.E.M.		
	Rat	Rabbit	Mouse
Total Polar Metabolites			
Noninduced	245 ± 15	625 ± 21	595 ± 64
Phenobarbital	3970 ± 154*	3654 ± 178*	3830 ± 320*
3-Methylcholanthrene	981 ± 97*	239 ± 15*	1956 ± 25*
GSH-Bromobenzene Conjugate**			
Noninduced	25 ± 6	47 ± 4	39 ± 3
Phenobarbital	489 ± 29*	306 ± 13*	492 ± 85*
3-Methylcholanthrene	133 ± 12*	15 ± 3*	146 ± 9*

* $p < .01$, when compared with Noninduced.

** Not corrected for recovery.

Significance to Biomedical Research and the Program of the Institute: These findings indicate that chemicals known to alter the metabolizing capacity of the microsomal drug oxidizing enzymes need not effect each species to the same degree or even in the same qualitative direction.

Proposed Course of Project: Microsomes from other tissues, such as the kidney will be studied in relation to the effects of phenobarbital and 3-MC on bromobenzene metabolism.

Honors and Awards: None

Publications: None

Serial No. NHLI- 211
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Requirements for the Formation of Glutathione Conjugate of Bromobenzene in vitro

Previous Serial Number: None

Principal Investigators: Dr. I. Glenn Sipes
Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: To investigate the possibilities that: 1) rat liver microsomes can convert bromobenzene from a chemically inert aromatic hydrocarbon into a highly reactive, electrophilic metabolite; 2) this highly reactive metabolite can covalently alkylate reactive groups of potent, endogenous nucleophils (e.g., glutathione); 3) compounds that alter the microsomal drug metabolizing enzymes will alter the in vitro metabolism of bromobenzene.

Methods Employed: ^{14}C-bromobenzene was incubated with isolated liver microsomes (2 mg/ml) from control or phenobarbital pretreated rats. This incubation mixture (pH 7.0) also contained dialyzed hepatic soluble fraction (0.2 ml/ml); glutathione (3 mM); a NADPH-generating system (0.124 mM NADH, 2 mM nicotinamide, 0.2 mM NADP, 2 mM glucose-6-PO_4, and 1 unit/ml glucose-6-phosphate dehydrogenase). Incubations were carried out at 37°C for a period of 4 min. Inhibitors of the microsomal drug metabolizing enzymes were added directly to these incubation mixtures. The incubations were terminated by addition of heptane or glacial acetic acid. Polar metabolites were determined by counting an aliquot of the aqueous layer following 3 heptane extractions. The bromobenzene-GSH conjugates were separated by adsorption onto charcoal followed by paper chromatography.

Major Findings: Rat liver microsomes possess an ability to metabolize bromobenzene. This was evident from the appearance of nonheptane extractable (polar) metabolites of bromobenzene. Further purification of the aqueous

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phase via paper chromatography indicated the formation of metabolite with an R_f of 0.4 to 0.5, which reacted with ninhydrin. A peak with the same R_f value was found when ^{35}S -GSH and unlabeled bromobenzene were used instead of ^{14}C-bromobenzene and unlabeled GSH. These results indicate the formation of GSH-bromobenzene conjugate.

Omission of soluble fraction from the incubation mixture decreased the amount of bromobenzene-GSH conjugate formed by 97%. Moreover, the conjugate did not form when the microsomes, NADPH-generating system, or O_2 were omitted from the incubation media. When the complete system was incubated in a CO-O_2 (9:1) atmosphere, the formation of the conjugate was reduced by 94%.

When liver microsomes were isolated from rats pretreated with phenobarbital or 3-methylcholanthrene (3-MC), the bromobenzene metabolizing activity was markedly enhanced; phenobarbital being the more potent inducer. The overall rate of metabolism was enhanced since both the total polar metabolites and bromobenzene-GSH conjugate increased the same degree. A 16-20 fold increase was obtained with phenobarbital induction while a 4-6 fold increase was obtained when 3-MC was used as an inducer of microsomal enzyme system. When SKF 525-A was administered to rats (75 mg/kg, i.p.) 2 hr prior to isolation of microsomes, the bromobenzene metabolizing capacity of these microsomes was reduced by 75%.

SKF 525-A and metyrapone, when incubated with liver microsomes, reduced the bromobenzene metabolizing capacity of the microsomes. SKF 525-A was slightly more potent inhibitor than metyrapone (I_{50} for SKF 525-A 1.5×10^{-5} M, metyrapone 2.5×10^{-5}M).

Significance to Biomedical Research and the Program of the Institute: These finding support the contention that bromobenzene, a relatively inert aromatic hydrocarbon, is converted to a highly reactive intermediate (possibly an epoxide) by a cytochrome P-450 enzyme in liver microsomes. This reactive intermediate can serve as an alkylating or electrophilic agent which will react with various nucleophilic substances. Such a reaction can result in the formation of a covalent bond and may explain the hepatic necrosis produced by bromobenzene and other aromatic hydrocarbons.

Proposed Course of Project: Clinically used drugs, especially those that produce a high incidence of tissue necrosis or tissue inflamation should be investigated for possible similarities in metabolism to highly reactive intermediates. If these intermediates are formed, it is highly probable that many of their toxic effects can be explained by a reaction of this metabolite with reactive sites on endogenous molecules.

Honors and Awards: None

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Serial No. NHLI-211

Publications: Brodie, B.B., Reid, W.D., Cho, A.K., Sipes, G., Krishna, G. and Gillette, J.R.: Possible mechanism of liver necrosis caused by aromatic organic compounds. Proc. Nat. Acad. Sci. 68: 160-164, 1971.

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Serial No. NHLI- 212
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Metabolism of ^{14}C-Bromobenzene Metabolism by Rabbit Kidney Microsomes

Previous Serial Number: None

Principal Investigator: Dr. I. Glenn Sipes
Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: It has been postulated by this laboratory that the active metabolite responsible for tissue damage is the epoxide of bromobenzene. Extensive studies on the metabolism of bromobenzene by liver microsomes indicate the formation of an epoxide that can react with glutathione (GSH) to produce a bromobenzene-GSH conjugate. Therefore measurement of the conjugate gives an idea of the amount of bromobenzene epoxide formed. Utilizing the same theory and methodology, the metabolism of bromobenzene by kidney microsomes was investigated.

Methods Employed: Microsomes were isolated from male rabbits (control and phenobarbital pretreated) following homogenization of the kidneys in cold Tris-KCl buffer (pH 7.4). After incubation of the microsomes with ^{14}C-bromobenzene (0.5 mM), the bromobenzene-GSH conjugate was isolated as previously described.

Major Findings: The following table summarizes the current findings on the metabolism of ^{14}C-bromobenzene by rabbit kidney microsomes.

EFFECTS OF PHENOBARBITAL PRETREATMENT ON THE METABOLISM OF ^{14}C-BROMOBENZENE BY RABBIT KIDNEY MICROSOMES

	n	picomoles/min/mg microsomal protein ± S.E.M.
Total Polar Metabolites		
Noninduced	3	85 ± 10
Phenobarbital	3	333 ± 15*

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GSH-Bromobenzene Conjugate**

Noninduced	5	6 ± 1
Phenobarbital	5	34 ± 3*

* $p < .01$, when compared with Noninduced

** Not corrected for Recovery

It is evident that phenobarbital pretreatment markedly enhances the bromobenzene metabolizing capacity of rabbit kidney microsomes. This is similar to findings for liver microsomes. It appears that the bromobenzene metabolizing capacity of the kidney microsomes is about 10% of that rabbit liver microsomes.

Significance to Biomedical Research and the Program of the Institute: Tissue damage produced by exposure to foreign compounds (including the controled administration of drugs) have always presented problems to the medical community. These findings indicate that there may be a relationship between the normal detoxification process present in the organism and the extent of tissue damage.

Proposed Course of Project: Since these are only preliminary findings, further study is necessary on the metabolism of bromobenzene and related compounds by kidney microsomes. Once an accurate, reproducible assay is accomplished for these agents, various drugs known to produce deleterious effects on the kidney will be studied.

Honors and Awards: None

Publications: None

Serial No. NHLI-213
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Differentiation of ATP and Cyclic AMP Pools in the Fat Cell
Previous Serial Number:	None
Principal Investigator:	Dr. Jay Moskowitz Dr. James P. Harwood Dr. Gopal Krishna
Other Investigators:	None
Cooperating Unit:	Dr. Moskowitz is a Research Associate in the Pharmacolog-Toxicology Program, NIGMS

Project Description:

Objectives: Adenosine 3',5'-monophosphate (cyclic AMP) has been implicated in the mediation of numerous hormonal effects. Because of some discrepancies between the levels of cyclic AMP within the cell and physiologically mediated effects of cyclic AMP (such as lipolysis) a multi-pool hypothesis has been suggested. We have utilized two methods of determining cyclic AMP levels and two methods of labeling ATP pools in order to characterize different pools of cyclic AMP.

Methods Employed: Male Sprague-Dawley rats (200-240 g) were used in all experiments: a) preparation of labeled fat cells in situ: Rats were anesthetized by intraperitoneal administration of pentobarbital (40 mg/kg). Testes and epididymal fat pads were exposed by an incision in lower right abdominal quarter and allowed to hang beneath the animal into a lucite incubation bath similar to that designed by Stein and Stein (Biochim. Biophys. Acta 54: 555, 1962). Each fat pad was allowed to incubate in 1 ml of KRP-buffer containing 10 uC_1 (4.3×10^{-7}M) of ^{3}H-adenine. After incubation the pad was placed back into the peritoneal cavity and the animal was sutured. b) Preparation of cells in vitro: Epididymal fat pads were removed from either preoperated (above) or normal animals and the cells were isolated by the method of Rodbell (J. Biol. Chem. 239: 379, 1964). Intracellular adenine nucleotides were labeled by preincubation with ^{14}C-adenine (1 MC_1/ 2×10^{-5}M). c) Cyclic AMP determination: ^{3}H-cyclic AMP, and ^{14}-cyclic AMP were isolated and assayed by the method of Krishna (J. Pharmacol. Exp. Ther. 163: 379, 1968). Unlabeled cyclic AMP was determined by the Gilman protein

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kinase binding assay (Proc. Nat. Acad. Sci. 67: 305, 1970). d) ATP determinations: ATP was determined in a Dupont luminescent Biometer by use of a luciferin-luciferase reaction.

Major Findings: a) ^{3}H-cyclic AMP, ^{14}C-cyclic AMP and total cyclic AMP in isolated fat cells are increased in response to norepinephrine in a dose dependent fashion (10^{-7}-10^{-4}M). The increase in the ^{3}H, ^{14}C and total cyclic AMP are qualitatively similar. b) Adenine prelabeled cells whether incubated in vivo for 7 days or in vitro for 15 minutes respond to norepinephrine (10^{-4}-10^{-7}) in a qualitatively similar fashion. c) The ratio of the specific activity of cyclic AMP divided by the specific activity of ATP for either isotope (e.g., ^{3}H-cyclic AMP/total cyclic AMP divided by ^{3}H-ATP/total ATP) increases with increasing dose of norepinephrine. Without the addition of catecholamine this ratio is 0.1. By increasing the dose of norepinephrine this ratio reaches a maximum of 0.4. The ratio never reaches 1 which would be indicative of a single pool of ATP contribution to cyclic AMP formation. The finding that this ratio increases towards 1.0 with an increase of the dose of norepinephrine, suggests that the radiolabeled pool of ATP is utilized to a greater extent in the formation of cyclic AMP when there is greater cell stimulation with norepinephrine. This increase in cell activity is reflected by the stimulation of glycerol release from the fat cell. d) Insulin will inhibit the dose response of the fat cells prelabeled and incubated for 7 days in vivo. Onset of this inhibition, however, is delayed for 3 minutes before the effect of 10 μU/ml of insulin is observed. This inhibition is observed in the total cyclic AMP levels as well as the labeled cyclic AMP. Cells incubated for varying times (1 minute intervals to 10 minutes) are observed to produce cyclic AMP at a linear rate for the first 3 minutes. The rate than appears to level off from the fourth to the sixth minute and then the levels of cyclic AMP decline. The rate of decline of cyclic AMP after the addition of insulin is greater than the decline observed with norepinephrine alone.

Significance to Biomedical Research and the Program of the Institute: These results suggest that the ATP contributing to the formation of cyclic AMP is not from a single pool. Since many hormones exert these actions on receptor cells through a mechanism involving cyclic AMP, this research will further elucidate the mechanism of hormone action on specific functions of the cell.

Proposed Course of Project: Further studies will be planned to study the specific pools of cyclic AMP that initiates the hormone included in cellular response.

Publications:

Moskowitz, J., Harwood, J.P., Reid, W.D. and Krishna, G.: The interaction of norepinephrine and prostaglandin E_1 on the adenyl cyclase system of human and rabbit blood platelets. Biochim. Biophys. Acta 230: 279-285, 1971.

Krishna, G., Moskowitz, Dempsey, P. and Brodie, B.B.: The effect of norepinephrine and insulin on brown fat cell membrane potentials. Life Sci. 9: 1353-1361, 1970.

Moskowitz, J., Harwood, J.P., Forn, J., Krishna, G., Rodgers, B. and Morrow, A.: The effect of norepinephrine and prostaglandin E_1 on adenosine 3',5'-monophosphate formation in isolated pericardial fat cells of man. Nature, in press.

Serial No. NHLI-214
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Bromobenzene Toxicity

Previous Serial Number: None

Principal Investigators: Dr. Michel Eichelbaum
Dr. Gopal Krishna

Other Investigators: Dr. Watson Reid

Cooperating Unit: Dr. Eichelbaum holds a Geigy Fellowship

Project Description:

Objectives: Previous studies in this laboratory have shown that the hepatotoxicity of bromobenzene is mediated through the binding of metabolites with tissue macromolecules.

The purposes of the studies carried out are: 1. to prove that covalent binding to tissue macromolecules (probably proteins) occurs; 2. to study the subcellular distribution of this irreversible binding; 3. to isolate the proteins and determine their molecular weight, to which the metabolites of bromobenzene are attached; and 4. to isolate and to elucidate the structure of the metabolites and amino acids, which form this covalent linkage.

Methods Employed: Male Sprague Dawley rats weighing 160-250 grams were pretreated on three successive days with phenobarbital (80 mg/kg) and injected on the fourth day with 0.15 g or 0.9 g/kg of ^{14}C-bromobenzene. Five hr later the animals were killed, the liver removed and homogenized in Tris KCl buffer. The 10,000 g pellet, the 105,000 g pellet (microsomes) and supernatant were prepared by differential centrifugation.

After precipitation of the fractions with TCA and filtration, the residue was stored for at least 24 hr in 40 volumes of methanol ether (3:1) at 4°C. The precipitate was filtrated, the residue placed in an extraction thimble and extracted for 48 hr with methanol and diethylether (3:1) at 60°C in a soxhlet apparatus. The residue was divided into two parts.

One part was dissolved in 1/15M phosphate buffer of pH 7.4, containing 1% SDS and subjected to gel chromatography on Sephadex G-100 [55 x 3]. The other part of the residue was dried in a dessicator over drierite. The resulting powder was then incubated in 0.2M phosphate buffer and 0.0015M $CaCl_2$ of pH 7.8 with pronase at 37°C for 96 hr. For the separation of the

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amino acids - bromobenzene metabolite ligand ion exchange chromatography and high voltage electrophoresis were used.

Major Findings: In experiments with livers from mice a striking difference between control and phenobarbital pretreated animals was found. The amount of covalently bound material was 3-4 times higher in the phenobarbital pretreated animals. This corresponds very well with the increase in the extent of the liver necrosis produced by phenobarbital. The table shows the covalent binding of bromobenzene to proteins in various subcellular fractions of rat liver:

	Bromobenzene Bound nmoles/mg Protein	
Fraction	0.15 g/kg (1 mmole/kg)	0.9 g/kg (6 mmole/kg)
Nuclear and Mitochondrial	0.5	7.8
Microsomal	2.2	14.0
Cytosol	1.25	6.6

By far the highest concentration of bound material is observed in the microsomes. The protein fractions when chromatographed on a Sephadex G-100 column gave two protein peaks containing the radioactivity. One was a low molecular weight protein of approximately 3,000. The material remaining in the protein after the extraction procedure described above is bound covalently to the proteins is based on the following facts: 1. The procedure used to prove covalent binding is same used by other authors to demonstrate covalent binding. 2. Aliquots of the protein hydrolysates were extracted at different pH's (1, 6 and 12) with ether. No C^{14} activity could be extracted in ether. Since all known metabolites of bromobenzene, namely (bromo)-phenol, dihydrodiol, -catechol and -mercapturic acid, are extractable at these pH's in ether, the metabolites of bromobenzene present in the protein may be bound covalently to the amino acids. This view is further supported by the fact that after drastic hydrolysis of aliquots of the protein with 6N HCl or 5N NaOH for 24 hr at 110°C, only 10-15% of the C^{14} activity were extractable in ether at pH 1.

The separation of the amino acids by high voltage electrophoresis was unsuccessful, since the amounts of amino acids to be applied on the paper to get measurable C^{14} activities were so great that the resolution of various amino acids became very deficient. Using a ligand exchange chromatography 3 amino acid peaks containing C^{14} could be eluted. These peaks appear to coincide with the cysteine, methionine and cysteic acid peaks.

Further studies to elucidate these radioactive amino acid peaks by mass spectrometry and to isolate some specific proteins containing covalently

linked bromobenzene are under progress.

Significance to Biomedical Research and the Program of the Institute: It has been proposed that bromobenzene causes hepatic necrosis through an active metabolite which then covalently binds to some macromolecules in the liver. The isolation of a protein containing bromobenzene covalently bound to it lends a strong support to the hypothesis that bromobenzene produces tissue lesions by formation of a highly reactive metabolite, probably an epoxide which then combines with cysteine and methionine of some macromolecules in the liver cell which ultimately leads to tissue necrosis.

Proposed Course of Project: In order to investigate the mechanism by which covalently linked bromobenzene to a macromolecule, it is necessary to isolate and characterize a specialized protein in the macromolecule which might play an important role in maintaining the integrity of the cell. This type of work would be extended to other drugs like phenylbutazone, diphenylhydantoin which occasionally produces liver damage.

Honors and Awards: None

Publications: None

Serial No. NHLI- 215
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	A Simple and Sensitive Method for the Measurement of Endogenous Cyclic AMP
Previous Serial Number:	None
Principal Investigator:	Dr. Gopal Krishna Dr. James P. Harwood Dr. Jay Moskowitz
Other Investigators:	Miss Ethel Boykins
Cooperating Units:	Dr. Moskowitz is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: The importance of adenosine 3',5'-monophosphate (cyclic AMP) in the mechanism of action of many hormones necessitates a simple and sensitive assay for the cyclic nucleotide in small tissue samples. Many methods for the assay of cyclic AMP are described in the literature which have one or more of the following disadvantages - multistage enzymatic conversions; preparation and purification of enzymes (e.g., phosphorylase and phosphorylase kinase); large tissue sample required for assay and requirement of extraction and concentration of the cyclic nucleotide; lack of sensitivity; slow, time consuming procedures which limit the number of assays which can be performed in one day.

The radio immunoassay technique for the estimation of plasma insulin, introduced by Berson and Yalow, has been adapted to the measurement of other circulating polypeptide hormones. The availability of a protein which specifically binds cyclic AMP enables the technique to be used for the measurement of tissue cyclic AMP levels. Two such proteins are available; protein kinase from skeletal or cardiac muscle, and a cyclic AMP antibody prepared by injection of a cyclic AMP-protein conjugate. The method is based upon competitive binding to the protein between radiolabeled cyclic AMP and nonradioactive cyclic AMP. As cold cyclic AMP is added to the system (standards or sample) the binding of labeled cyclic AMP is reduced in proportion to the amount added. The protein-cyclic AMP complex is passed over a membrane filter, which is then washed and counted. From a standard curve,

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the amount of cyclic AMP in a sample is readily determined.

Methods Employed: Protein kinase was purified from beef muscle up to and including the stage of chromatography on DEAE cellulose, as described by Miyamoto, Kuo and Greengard (J. Biol. Chem. 244: 6395, 1969). Two protein peaks are obtained from the column; the second peak was collected and used for the assay. The inhibitor (a small protein in skeletal muscle which inhibits protein kinase activity, but at the same time appears to increase the affinity of the protein kinase for cyclic AMP) was prepared from the same beef muscle by the method described by Appleman et al. (Arch. Biochem. Biophys. 116: 39, 1966). The assay is based on the procedure described by Gilman (Proc. Nat. Acad. Sci. 67: 305, 1970). The tissue (usually a 1 ml suspension of fat cells or platelets) was killed by the addition of 0.5 ml of 10% trichloroacetic acid (TCA). Following centrifugation, 0.2 ml aliquots of the supernatant were extracted three times with 2.5 ml of water-saturated ether. Any remaining ether was removed by placing in a warm water bath for a few minutes. In the assay 10 µl of the ether-extracted supernatant or standard was added to small disposable culture tubes. To these tubes were added 20 µl of a mixture consisting of equal parts of 1) protein inhibitor (1 mg/ml), 2) 50 mM sodium acetate/acetic acid buffer pH 4.1, 3) labeled ^{3}H-cyclic AMP (200 pmoles/5 x 10^{6} cpm in 1 ml), and 4) water. The reaction was started by the addition of 20 µl of the protein kinase, and the reaction allowed to proceed for not less than one hr at 0°C. After 1 hr, 1 ml of ice-cold 20 mM potassium phosphate buffer (pH 6.0) was added and after 3 to 4 min, the mixture carefully passed through a millipore filter. After washing with 10 ml of the same buffer (pH 6.0) the filter was dissolved in 1 ml of ethylene glycol monomethyl ether, fluor added, and counted in a liquid scintillation counter.

Major Findings: 1) The standard curve was constructed using standards ranging from 0.1 to 20.0 pmoles in 10 µl. When plotted on double logarithm paper, a straight line was obtained. Standards in duplicate or triplicate were run with each assay. 2) The sensitivity of the method is such that it will measure cyclic AMP from 0.1 to 20.0 pmoles in a 10 µl sample. 3) The assay can be performed on crude tissue extracts, thus avoiding the need for prior isolation of the cyclic nucleotide. 4) The assay has been applied to determine cyclic AMP concentrations directly in urine samples. Only 0.01 to 0.1 µl urine samples are required. 5) The assay is sufficiently rapid such that an experiment involving the assay of 100 samples for cyclic AMP can be completed in a regular day. 6) The assay has been applied to the measurement of total cyclic AMP levels in fat cells, platelets, brain and urine. In the experiments in platelets and fat cells simultaneous assays were carried out for total cyclic AMP, using the above described binding assay, and ^{3}H cyclic AMP from prelabeled ^{3}-ATP obtained on preincubation of cells within ^{3}H-adenine. In the case of fat cells and platelets there appears to be a parallelism in the formation of ^{3}H and total cyclic AMP.

Significance to Biomedical Research and the Program of the Institute: With the availability of a simple and sensitive method for cyclic AMP, it should be possible to measured the formation of cyclic AMP in response to

various hormones in a variety of cells. This method should enable us to study the turnover of cyclic AMP in tissues under a variety of conditions.

Proposed Course to Project: With the availability of a simple and sensitive assay for cyclic AMP in tissues, it should be possible to conduct *in vivo* and *in vitro* experiments to measure the turnover of cyclic AMP in various tissues. It is also proposed to use this method to study the adenyl cyclase enzyme using various ATP analogues which are not hydrolyzed by the ATPase.

Honors and Awards: None

Publications: None

Serial No. NHLI-216
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Use of the Isolated Liver Cell to Study Drug Metabolism

Previous Serial Number: None

Principal Investigator: Dr. Jay Moskowitz
Dr. I. Glenn Sipes
Dr. Gopal Krishna

Other Investigators: None

Cooperating Units: Dr. Moskowitz is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: To develop a method for the isolation of rat liver cells and to utilize these cells in the study of drug metabolism. While a great deal is known on the metabolism of tissue slices and microsomes, very little is known of isolated liver cells in metabolism of various drugs. Isolated liver cells may also serve in the study of mechanism of toxicity produced by Cl_4, bromobenzene and other hepatotoxins.

Methods Employed: The isolated cells were prepared by removing the entire rat liver and perfusing it immediately through the portal vein with Ca^{++} free HANKS solution containing 4% albumin equilibrated with $95/0.5/CO_2$. After the liver is free of blood cells the liver is perfused with Ca^{++} free HANKS (4% Albumin) solution containing 0.1% collagenase and hyaluronidase for 30 min 37°C. The liver is then dispersed and incubated for 15 more min in the HANKS buffer containing collagenase and by hyaluronidase. The cells are then washed and isolated as described by Berry and Friend (J. Cell. Biol. 43: 506, 1969).

Major Findings: The isolated liver cells have been shown to metabolize bromobenzene without any addition of cofactors. Though the cells metabolize bromobenzene to a much smaller extent than isolated microsome preparations, the cells can metabolize this compound at a linear rate up to 20 min. The liver cells isolated from liver of rat pretreated with phenobarbital show a greatly enhanced rate of metabolism of bromobenzene. The cells also can metabolize ethylmorphine, but again the rate of metabolism is very low.

Significance to Biomedical Research and Program of the Institute: Recent observations suggest a variation in some metabolic properties of individual cells according to concentration of cells. The rate of incorporation of amino acids into reticulocyte protein has been shown to vary inversely with the concentration of cells in a suspension (Bhargava et al., Biochem. J. 73: 247, 1959). The preparation of liver cells isolate each cell from its neighboring cell thus stopping intercellular dependence. The comparison of data using the isolated cell to that of slices and perfused intact liver will prove invaluable in assessing the metabolism of the liver.

A similar concentration effect has been observed with the respiration of spermatozoa (Bishop and Salisbury, Amer. J. Physiol. 180: 107, 1955). This effect is not due to nutrient being the limiting factor and it is possibly caused by intercellular reactions which may be related to growth organization.

Proposed Course of Project: To develop an improved method of isolating liver cells with minimal loss of ions and cellular components as well as a survey of drug metabolism.

Honors and Awards: None

Publications: None

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Serial No. NHLI-217
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Indomethacin-induced Ulcerations of the Gastrointestinal Tract

Previous Serial No.: None

Principal Investigators: Dr. Jerry R. Mitchell
Dr. James R. Gillette

Other Investigators: None

Cooperating Unit: Dr. Mitchell is a Research Associate in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Gastrointestinal ulcerations are produced by all anti-inflammatory drugs. This liability is so great that the most effective drugs such as indomethacin and phenylbutazone currently are restricted to patients with advanced rheumatoid or osteoarthritis or to short-term treatment of acute episodes of gouty arthritis.

Recently, the intestinal ulcerations produced by indomethacin in rats and dogs were shown to be prevented by ligation of the common bile duct. This suggests that metabolism of indomethacin by the liver may be responsible for the intestinal lesions. Since the parent indomethacin and not a metabolite is the active anti-inflammatory agent, the possibility of preventing indomethacin's intestinal toxicity by altering its metabolism but without decreasing its anti-inflammatory action has obvious clinical implications. Accordingly, we have attempted to define the mechanism of indomethacin-induced ulcerations of the intestine.

Methods Employed: An evaluation of the effects of piperonyl butoxide, a compound known to inhibit many hepatic microsomal drug-metabolizing enzymes, on ulcerations produced by indomethacin in adult male rats was carried out. Similar experiments in rats pretreated with 3-methylcholanthrene, which increases the rate of metabolism of a number of drugs, were done.

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Major Findings: Piperonyl butoxide (340 mg/kg, s.c., twice daily) administered concomitantly with indomethacin (4-12 mg/kg, s.c. daily) for two days markedly decreased the number of intestinal ulcers and intestinal perforations. 3-Methylcholanthrene moderately potentiated these toxic effects of indomethacin.

While less clear-cut, similar results were obtained when indomethacin was given intravenously (10-25 mg/kg) and the degree of ulceration examined 8 hr later.

These data support the concept that the intestinal toxicity of indomethacin is mediated by an active metabolite. Because 3-methylcholanthrene and piperonyl butoxide may alter biliary excretion of indomethacin (and thereby change its toxicity) proof of this postulate awaits an evaluation of these factors.

Significance to Biomedical Research and the Program of the Institute: If the intestinal ulcerations produced by indomethacin are mediated through an active metabolite, it should be possible clinically to prevent its toxicity without decreasing its anti-inflammatory properties by inhibiting its metabolism.

Proposed Course of Project: Studies will be continued to examine the role of drug metabolism in ulcer formation with particular emphasis on those factors which modify the toxic response.

Honors and Awards: None

Publications: None

Serial No. NHLI-218
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Bromobenzene-Induced Hepatic Necrosis: Species Differences and Protection by SKF 525-A.

Previous Serial Number: None

Principal Investigator: Dr. Jerry R. Mitchell

Other Investigators: Dr. Watson D. Reid
Dr. Jay Moskowitz
Dr. Gopal Krishna
Dr. Basil Christie

Cooperating Units: Drs. Jerry R. Mitchell and Jay Moskowitz are Research Associates in the Pharmacology-Toxicology Program, NIGMS.

Project Description:

Objectives: Previous work by this laboratory demonstrated that pretreatment of rats with phenobarbital potentiates the hepatic necrosis produced by bromobenzene and a number of other chemically inert halogenated aromatic hydrocarbons. It was postulated that the hepatotoxic effects of the aromatic hydrocarbons result from chemically active metabolites, presumably epoxides, formed in hepatocytes.

The use of species differences in drug metabolism for elucidating mechanisms of the pharmacologic and toxicologic action of many drugs is well established. Accordingly, the relationship between bromobenzene-induced liver necrosis and bromobenzene concentrations in plasma and liver of various species was studied in an effort to clarify the role played by drug-metabolizing enzymes in the hepatotoxicity produced by chemically inert organic compounds. In addition, preliminary experiments were carried out to evaluate the effects of SKF 525-A (diethylaminoethyl diphenylpropylacetate), a compound known to inhibit many hepatic microsomal drug-metabolizing enzymes, on bromobenzene metabolism and liver necrosis.

Methods Employed: Adult male mice, rabbits, hamsters and guinea pigs were obtained from NIH stock. Hens, toads, frogs, neonatal and adult Sprague-Dawley rats and several strains of adult male mice (C 57/Blk 6, A/StC, DBA, Balb/C, C3H) were obtained commercially. All species were given various doses of bromobenzene i.p. and killed 24 hr later. SKF 525-A (75 mg/Kg, i.p.) was injected 1 hr before bromobenzene administration and at intervals of 8 hr thereafter. Paraffin sections of liver were prepared and stained with

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periodic acid - Schiff reagent (PAS) to stain glycogen and with hematoxylin to counterstain the nuclei. Concentrations of bromobenzene in liver and plasma were assayed on a gas-liquid chromatograph equipped with a ^{63}Ni ionization detector.

Major Findings:

	Hepatic Necrosis after Bromobenzene 1 ml/Kg i.p.	24 Hr Bromobenzene Concentration Plasma (ug/ml)	Liver (ug/g)
NIH Mouse	Extensive	1.0 ± 0.1 (4)	18 ± 4 (4)
Rat	Extensive	2.8 ± 0.3 (7)	26 ± 3 (7)
Rabbit	Moderate	-	35 ± 12 (3)
Hamster	Moderate	3.2 ± 0.5 (3)	42 ± 9 (3)
Neonatal Rat	None	3.8 ± 0.3 (6)	89 ± 24 (6)
Hen	None	6.9 ± 1.1 (7)	214 ± 39 (7)
Toad	None	-	151 ± 34 (6)
Frog	None	-	1701 ± 106 (6)
SKF 525-A-Treated Rat	None	14.4 ± 0.5 (5)	149 ± 8 (5)

Those species which had the highest concentrations of bromobenzene in plasma and liver showed no hepatic damage while those with the lowest concentrations suffered extensive necrosis. Furthermore, blockade of metabolism by SKF 525-A resulted in high concentrations of bromobenzene in plasma and liver yet prevented the hepatic necrosis.

These data demonstrate that bromobenzene itself does not produce cellular damage; a toxic metabolite, presumably bromobenzene epoxide, must be the chemically active agent.

Significance to Biomedical Research and the Program of the Institute: These results clearly establish the crucial role played by hepatic microsomal enzymes in activating some chemically inert compounds to highly reactive metabolites which can form covalent linkages with tissue macromolecules and thereby produce tissue lesions. A similar activation of many therapeutic drugs to reactive forms which combine with tissue components to produce tissue damage directly or to create drug-tissue antigens which lead to drug allergy is highly probable.

Proposed Course of Project: Terminated.

Honors and Awards: None

Publications: None

Serial No. NHLI- 219
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Dimethylbenzanthracene-Induced Bone Marrow, Spleen, Testis and Gut Necrosis

Previous Serial Number: None

Principal Investigator: Dr. Jerry R. Mitchell
Dr. Amin Suria

Other Investigators: Dr. Bitten Stripp
Dr. David Jollow

Cooperating Units: Dr. Mitchell is a Research Associate in the Pharmacology-Toxicology Program, NIGMS.

Project Description:

Objectives: In looking for other chemically inert compounds which cause bone marrow injury in a reproducible, dose-response manner, we have found a carcinogen dimethylbenzanthracene (DMBA) to be very potent after a single intravenous injection. In addition to its bone marrow damage, DMBA also produced tissue lesions in the spleen, testis and gut. A study has been initiated to define the mechanism by which this chemically inert compound causes tissue damage and to determine whether covalent bonding of DMBA to tissue macromolecules is necessary for manifestation of toxicity.

Methods Employed: Rats or mice were injected i.v. with DMBA and killed 5 hr later. Five min before death ^{3}H-thymidine (specifically labeled) was given i.v. Liver, lung, bone marrow, spleen, gut and testes were removed, homogenized and assayed for content of ^{3}H-thymidine per μg of DNA, as a parameter of DNA synthesis. (Preliminary investigations demonstrated that this parameter reliably reflected tissue damage seen histologically at later time intervals). 7,8-Benzoflavone, which inhibits DMBA's metabolism in vitro, was given to some animals 1 hr before administration of DMBA.

In other experiments ^{14}C-DMBA was given to mice and the above-mentioned tissues were examined 5 hr later for chemical evidence of covalent bonding of DMBA to tissue macromolecules. The influence of benzoflavone on this covalent bonding of DMBA was also examined.

Major Findings: DMBA inhibited DNA synthesis in the bone marrow, spleen, gut and testis by 80% but only by 25% in the liver and lung. Furthermore, DNA synthesis was also inhibited in the gut by 80% 24 hr later when necrosis was seen histologically. However, neither inhibition of DNA synthesis nor

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necrosis were found in the liver 24 hr after administration of DMBA.

In contrast, the inhibition of DNA synthesis by DMBA was prevented in the animals pretreated with benzoflavone.

Other studies demonstrated covalent bonding of ^{14}C-DMBA to macromolecules in all tissues after i.v. administration. No bonding of DMBA to tissue occurred when DMBA was added to tissue homogenates in vitro. Pretreatment with benzoflavone decreased the covalent bonding of DMBA to tissue macromolecules by 60-80%.

Significance to Biomedical Research and the Program of the Institute: These data suggest that an active metabolite of DMBA, presumably DMBA epoxide, is responsible for the tissue lesions and that covalent bonding of the chemically active metabolite to tissue macromolecules may be the mechanism mediating the damage. Since most therapeutic drugs also are chemically inactive, these studies raise the possibility that a similar activation of these agents by microsomal enzymes may explain how the drugs occasionally produce tissue lesions in the liver, kidney, bone marrow or skin of patients. In addition, the results could be of considerable importance in understanding the mechanisms by which polycyclic aromatic hydrocarbons cause cancer.

Proposed Course of the Project: Further delineation of the role of microsomal enzymes in DMBA's toxicity and of the necessity of bonding of DMBA to specific tissue macromolecules (DNA vs RNA vs protein) for manifestation of tissue damage.

Honors and Awards: None

Publications: None

Serial No. NHLI-220
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanisms of Drug-Induced Bone Marrow Damage

Previous Serial Number: None

Principal Investigator: Dr. Jerry R. Mitchell
Dr. James R. Gillette

Other Investigators: Dr. David Jollow
Dr. Nicola Zampaglione
Dr. Amin Suria

Cooperating Units: Drs. Jerry R. Mitchell and Nicola Zampaglione are Research Associates in the Pharmacology-Toxicology Program, NIGMS.

Project Description:

Objectives: Bone marrow (BM) injury is one of the most serious complications of drug therapy. Among currently marketed drugs, more than 80 are capable of causing some type of BM damage. In an effort to gain an insight into the mechanisms by which drugs damage BM, we approached the problem by beginning with a chemically inert compound, benzene, which causes BM injury in a reproducible, dose-response manner.

Methods Employed: Although some reports suggest that benzene is directly toxic to BM, most workers feel that benzene's toxicity is produced by its phenolic metabolites. To evaluate these possibilities, benzene (0.5 to 1.5 ml/Kg, s.c.), phenol (0.25 to 0.75 g/Kg, s.c.), catechol and hydroquinone (each 25 to 75 mg/Kg, s.c.) were administered daily in divided doses to adult male rats for one week. Aortic blood was obtained for a complete blood count with differential, and both femurs were removed for a BM nucleated cell count and for evaluation of BM nucleic acid and protein synthesis by *in vitro* incorporation of ^{3}H-thymidine and a ^{14}C-amino acid mixture.

Major Findings: Rats receiving benzene (1 ml/Kg, s.c.) daily for 1 week developed aplastic anemia. In contrast, although the largest doses of the phenolic metabolites killed approximately 50% of the rats, no hematopoietic toxicity was found. In additional studies, benzene was found to be toxic only in high concentrations (>500 µg/ml) to BM cells cultured in McCoy's media for 3 hr to 8 days. Also, piperonyl butoxide and phenobarbital treatment protected against benzene-induced aplastic anemia. Preliminary metabolism studies, however, indicated that these compounds alter the rate of metabolism of benzene very little. Since chronic treatment with benzene is

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necessary to produce aplastic anemia, the effects of chronic benzene administration (3 days) on the pattern of urinary metabolites of ^{14}C-benzene was examined. Chronic treatment with benzene resulted in the appearance of two new metabolites, tentatively identified as phenyldihydrodiol and phenylmercapturic acid. Concomitant administration of phenobarbital or piperonyl butoxide inhibited the formation of these metabolites.

An attempt was made to expand the study to include the halogenated benzenes. However, bromobenzene, o-dibromobenzene, p-dibromobenzene and fluorobenzene failed to produce bone marrow damage.

In summary, it is concluded that benzene's metabolism by hepatic microsomal enzymes is requisite for its BM toxicity. Although identification of the active metabolite has not as yet been completed, preliminary results suggest that benzene itself may induce a new metabolic pathway which produces a toxic metabolite, possibly an epoxide since dihydrodiol appears in the urine.

Significance to Biomedical Research and the Program of the Institute: 1. The finding that a compound fails to cause tissue damage after acute administration but after chronic treatment induces a new metabolic pathway which produces a toxic metabolite has immense biomedical significance. If this phenomenon is found with other drugs, the many failures to define the mechanism of drug-induced tissue lesions after acute administration of the drugs may be explained. 2. The data also indicate that there may be more than one biochemical pathway by which phenols are formed by the liver, a finding of considerable biochemical interest for drug metabolism research.

Proposed Course of Project: 1. Identification of the active metabolite of benzene responsible for BM injury. 2. Evaluation of the possible role of self-induction of toxic pathways of metabolism for other drugs after chronic administration. 3. Delineation of the number of enzymic pathways involved in the metabolism of aromatic drugs to phenols and clarification of the relative role played by each pathway in producing tissue damage.

Honors and Awards: Invited Speaker, National Academy of Sciences Drug Research Council.

Publications: None

Serial No. NHLI-221
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Hepatotoxicity of Halogenated Aryl Hydrocarbons. 2. Effect of Phanobarbital and 3-Methylcholanthrene Induction on the Hepatotoxicity and the Pattern of Metabolism of Bromobenzene

Previous Serial Number: None

Principal Investigators: Dr. David Jollow
Dr. Nicola Zampaglione

Other Investigators: Dr. Maynard Hamrick
Dr. Bitten Stripp
Dr. James R. Gillette

Cooperating Units: Drs. Maynard Hamrick and Nicola Zampaglione are Research Associates in the Pharmacology-Toxicology Program, NIGMS

Project Description:

Objectives: Treatment of rats with phenobarbital and 3-methylcholanthrene is known to cause induction of a wide variety of microsomal enzymes in the liver. The hepatotoxicity of bromobenzene in rats is enhanced by phenobarbital and decreased by 3-methylcholanthrene pretreatment. Since the hepatotoxicity is believed to be due to an intermediate in bromobenzene metabolism (bromobenzene epoxide) studies were undertaken to determine whether induction by these agents caused differences in the pattern of bromobenzene metabolism.

Methods Employed: 1. Biological half-life of bromobenzene. Bromobenzene was administered intraperitoneally in the dose levels used for the toxicology experiments (normal and 3 MC induced rats received 1 ml/Kg in sesame oil, Pb induced rats received 0.15 ml/Kg in sesame oil), and the plasma levels of bromobenzene were determined. When the plasma levels were maximal, carrier-free ^{14}C-bromobenzene were injected i.v. and the decay of label followed. The half-life was determined for the whole animal, plasma, liver, brain, muscle and epididymal fat pad. 2. Urinary Metabolic Profile. Bromobenzene (2 μc, 0.2 ml/ml sesame oil/200 g rat) was injected i.p. and the urine was collected at 3, 6, 12, 24 and 48 hr. The urinary metabolic profile was determined, after a glucuronidase and sulphatase digestion, by thin layer chromatography.

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Major Findings: 1. In untreated rats the biological half-life of i.v. administered ^{14}C-bromobenzene was 11 min. The major metabolites obtained 48 hr after administration were bromophenyl mercapturic acid (58%) and bromophenol (25%). Bromocatechol (10%) and bromobenzenedihydrodiol (8%) were minor metabolites. 2. Phenobarbital pretreatment of rats caused an increase in rate of ^{14}C-bromobenzene metabolism (biological half-life, 7 min) but no significant alteration in the urinary metabolite profile. 3. 3-Methylcholanthrene caused no induction of metabolism compared with the untreated rat but did alter the urinary metabolite profile; bromobenzenedihydrodiol becoming a major metabolite at the expense of the mercapturic acid and bromophenol. Data on urinary metabolites obtained at shorter time points (3 and 6 hr) suggested a larger output of the diol. 4. These data raise the possibility that the protection from bromobenzene hepatotoxicity afforded by 3-methylcholanthrene is not due to inhibition of metabolism but may be due to increased utilization of the epoxide intermediate by increased hydrase activity in the liver cell.

Significance to Biomedical Research and the Program of the Institute: These studies emphasize that a variety of factors in drug metabolism may influence the toxic manifestations of drug therapy and that these factors may be influenced by prior exposure to drugs or other environmental conditions.

Proposed Course of Project: Studies on the mechanism of bromobenzene metabolism will be continued with emphasis on the variety of metabolic pathways and the factors influencing the distribution of the epoxide intermediate into these pathways.

Honors and Awards: None

Publications: None

Serial No. NHLI-222
1. Chemical Pharmacology
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Hepatotoxicity of Halogenated Aryl Hydrocarbons. 1. Studies on the Relationship of Glutathione to the Hepatotoxicity of Bromobenzene

Previous Serial Number: None

Principal Investigators: Dr. David Jollow
Dr. James R. Gillette

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Induction of metabolism by pretreatment of rats with phenobarbital potentiates bromobenzene toxicity, while simultaneous administration of bromobenzene with SKF 525-A, an inhibitor of metabolism, blocks toxicity. Consideration of the nature of the urinary metabolites has led to the postulation of bromobenzene epoxide as an intermediate in the metabolic pathway. The epoxide intermediate could act as an arylating agent for cellular macromolecules, resulting in alteration in their cellular function and ultimately cell death. In vitro studies have established that isolated liver cell fractions catalyze the conjugation of reduced glutathione (GSH) with aryl epoxides. The concept of the aryl epoxide as the hepatotoxin indicates that liver GSH should oppose the toxic effect in vivo and that hepatotoxicity should occur only after depletion of GSH from the liver cell.

The present studies were undertaken to answer two questions: 1. Does administration of bromobenzene cause depletion of liver GSH levels; and 2. Does pretreatment of rats with agents known to change the degree of hepatotoxicity cause similar change in GSH depletion from the liver.

Methods Employed: Standard biochemical and pharmacological techniques were employed.

Major Findings: 1. Bromobenzene administration (1 ml/Kg, i.p.) to a normal rat caused a rapid and extensive loss of liver glutathione. Liver glutathione levels reached a nadir of about 20% of the initial level, 220-240 min after intraperitoneal injection. The half-life of glutathione disappearance in the normal rat was found to be 60 ± 8 min. The loss of glutathione was not due to conversion to the oxidized form but represented a net loss of glutathione from the hepatocytes. 2. Pretreatment of rats

with phenobarbital dramatically potentiated the hepatotoxicity of bromobenzene (0.15 ml/Kg, i.p.) and caused an equally dramatic decrease in the half-life of GSH disappearance (< 25 min). Comparison of glutathione disappearance with plasma bromobenzene levels suggested that the rate limiting step in glutathione disappearance was the uptake of bromobenzene from the peritoneal cavity. 3. After depletion of glutathione the level in the liver cells remains low for 5-16 hr and then increases. Elevation of cellular glutathione only becomes marked when liver bromobenzene levels drop significantly. Liver GSH levels 24-48 hr after bromobenzene administration are significantly elevated (ca 200%) compared with the levels before bromobenzene administration. The rise in liver GSH occurs irrespective of the degree of centrolobular necrosis in the liver. 4. Simultaneous administration of SKF 525-A, which is known to decrease the rate of metabolism of bromobenzene and to protect from the hepatotoxicity, significantly slows depletion (T 1/2 100 min). 5. Dose response studies for glutathione disappearance after bromobenzene administration revealed that the dose of bromobenzene necessary to cause a 50% depletion of liver GSH is very much smaller 10-15%) than the dose necessary to cause centrolobular necrosis in the liver.

Conclusions: 1. The observed rapid depletion of GSH from the liver of normal rats treated with bromobenzene, the enhanced depletion of glutathione accompanying enhanced toxicity in phenobarbital pretreated rats, and the decreased rate of depletion accompanying protection by SKF 525-A, offers support for the postulated role of aryl epoxides as the toxic agent on administration of halogenated aryl hydrocarbons.

Significance to Biomedical Research and the Program of the Institute: The present studies are aimed at an understanding of the mechanism of drug-induced toxicity and at the factors which are concerned in the protection of the organism from the toxic effects. Such information is essential for design of drugs with minimal untoward effects.

Proposed Course Project: Studies will be continued to explore the relationship between glutathione depletion and bromobenzene hepatotoxicity.

Honors and Awards: None

Publications: None

Tech Development

Molecular Disease

Endocrinology

Chemistry

ANNUAL REPORT OF THE
ENDOCRINOLOGY BRANCH
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

The activities of the Endocrinology Branch have been concerned with four major areas:

A. Studies of calcium and phosphorus metabolism: The physical chemistry of the mineral phases of bone and of renal stones, with reference to factors controlling crystal formation and dissolution; studies of hormonal and other factors controlling bone formation and dissolution in vivo, and studies of parathyroid function in health and disease.

B. Studies of adrenal cortical function with special reference to biogenetic and physiologic control mechanisms and to hormonal aspects of hypertension.

C. Studies of renal function with especial reference to factors controlling sodium excretion and edema formation.

D. Studies of the properties of hormonal and other peptides: The chemical, physical and immunologic properties of the peptide hormones, parathyroid hormone and thyrocalcitonin, and of the protein moiety of very-low-density lipoprotein.

A. Studies of calcium and phosphorus metabolism.

1. Interactions of hormones at air-water interface - a model of hormone-membrane interaction? The studies of the effects of hormones at an air/mono-octadecyl phosphate-monolayer/water interface showed that in addition to thyrocalcitonin (which blocks uptake of Ca^{2+} by the monolayer), insulin and vasopressin have the same effect and also facilitate evaporation of water through the monolayer. In contrast, parathyroid hormone had little effect, and prostaglandin E_1 had none.

2. Ionic effects on bone crystal and on composition of the hydration shell. Kinetics of ^{45}Ca behavior were used to examine exchange of ions with hydration shell and crystal, both of bone and of synthetic calcium phosphates. Pyrophosphate, thought to inhibit calcification, was shown to inhibit precipitation of brushite ($CaHPO_4 \cdot 2H_2O$); fluoride, thought to inhibit bone resorption, was shown to reduce the concentration of Ca^{2+} and PO_4^{3-} in the hydration shell. These and other effects are being studied with bone mineral from patients with metabolic and unexplained bone disease.

3. The pathogenesis of calcium-containing renal stones. Precipitation of brushite ($CaHPO_4 \cdot 2H_2O$) shown to control the formation of stones on an organic or crystal matrix by incubating urine specimens with brushite or collagen. The activity product of Ca^{2+} by HPO_4^{2-} as measured needed only to exceed that for the solubility of brushite for stones to form or grow. Further, the same activity product was shown to control precipitation of brushite stones de novo; this required a product about three-fold that for growth on a nidus; precipitation

always began by removal of Ca^{2+} and HPO_4^{2-} in a one-to-one ratio, and the solid phase began as brushite. Since stone-forming patients required no lower a product for crystal formation than normals, it is concluded that inhibitors of calcification probably play little part in "protecting" these normals against stone formation.

These indices of a given patient's stone-forming tendency (on a nidus, as an activity product ratio , or de novo as a formation-product ratio) provided a logical basis for evaluation of treatment. Cellulose phosphate was then shown to lower the activity product ratio, and sodium phosphate to raise it. The sodium phosphate also increased the formation product ratio, possibly by increasing the pyrophosphate content of urine.

4. Effects of thyrocalcitonin. Thyrocalcitonin (TC) was shown to increase phosphorus clearance in thyroparathyroidectomized dogs but not in normal dogs; this effect was attributed to stimulation of parathyroid secretion.

TC was studied in the treatment of Paget's disease (in which it increased calcium excretion, possibly by increasing parathyroid activity), and in osteoporosis (where it had a similar effect).

A refined diagnostic tool was perfected to provide rapid measurement of calcium absorption by comparison of bone uptake of ^{47}Ca after an oral dose with that after the same dose given intravenously. It was thus shown that TC increases calcium absorption by the gut in patients with hypoparathyroidism: possibly a primary effect.

5. Metabolic bone disease. Repeated calcium infusions in man were further explored for therapeutic effects in patients with idiopathic osteoporosis. As with the earlier studies, some patients ("responders") showed prolonged positive calcium balance and relief of symptoms, whereas some ("non-responders") returned to negative calcium balance after a course of infusions. Calcium infusion given with TC was in no case more effective than calcium infusion alone. In the non-responders, the hormonal effects of calcium infusion were explored in detail. In the normal, an infusion of calcium produces (1) a decrease of plasma parathyroid hormone, (2) an increase in plasma TC. (It may be that these two changes are primarily responsible for the improvement in "responders".) (3) a decrease in urinary cyclic AMP and (4) hydroxyproline, and (5) a rise in urinary phosphorus followed by (6) a fall in urinary phosphorus. Changes 1, 3, 4 and 6 occur only when parathyroid secretion is decreased and remains low for 24 hours: they do not occur in hypoparathyroidism or hyperparathyroidism. Recent studies show that "non-responders" among osteoporotics include many subjects whose parathyroid function is "autonomous" vis-a-vis calcium infusion, in that changes 1, 3, 4 and 6 do not occur or are transient. Parathyroid adenoma or hyperplasia has thus been uncovered. To date, twenty patients with the syndrome of normocalcemic primary hyperparathyroidism have been studied and treated surgically - patients whose presenting sign was either such "autonomous" response to calcium infusion, nephrolithiasis, or both. The new test for absorption of calcium provided the first demonstration that parathyroid hormone increases calcium transport in vivo.

The effect of diuretics on urinary calcium and calcium balance was

re-explored. Thiazide decreased urinary calcium in normal subjects but not in patients with hypoparathyroidism: this suggests an action through the parathyroids. Furosemide had no such effect.

It was found that acidosis increases urinary calcium by decreasing tubular reabsorption, and that alkalosis decreases it. Gastrointestinal absorption of calcium increases with acidosis but this effect may be secondary to an increase in parathyroid hormone secretion, depending in turn on the increased urinary calcium loss. Sodium chloride loading markedly increased urinary calcium and also enhanced gastrointestinal absorption of calcium: the mechanism may be the same as that described for acidosis.

The Vitamin D metabolite, 25 hydroxycholecalciferol, was further studied. Two of three patients with resistant rickets showed prompt therapeutic response with rise of serum phosphorus. However, one of three patients with resistant rickets and one patient with hypoparathyroidism showed clear resistance to the metabolite. The last patient had renal failure; the results are thus consistent with the view that a further metabolite (? 1, 25 di-hydroxycholecalciferol), formed in the kidney, is necessary for the full action of Vitamin D. The syndrome of idiopathic hypercalcemia of childhood and infancy was studied to see whether TC deficiency was in part responsible for the hypercalcemia. In children recovered from the severe infantile form, no residual hormonal defect was uncovered. Parathyroid function was explored in five patients with cystic fibrosis of the pancreas, in whom the bones show a disease resembling osteoporosis. It was found that a state of hyperparathyroidism, "autonomous" vis-a-vis induced hypercalcemia, was present in all five; this could be ultimately secondary to malabsorption of calcium.

Current studies in the pathogenesis of metabolic bone disease include studies of the collagen of bone and skin. The solubility of collagen and its extent of hydroxylation was measured in normal bone and skin and compared to that in patients with osteoporosis studied before treatment and again after a course of calcium infusions. Results suggest that osteoporosis is characterized by an increased solubility and inadequate hydroxylation of collagen; in three patients studied thus far the results after treatment suggest that the collagen of bone may show maturation as measured by both these indices. The treatment of patients with penicillamine provides a "model" for the effect of penicillamine and other lathyrogens in reducing cross-linkage of collagen. Patients requiring penicillamine for the treatment of cystinuria are subjected to skin biopsy for measurement of collagen chemistry and then again after treatment with estrogens.

The development of radioimmunoassay for parathyroid hormone is underway. A successful radioimmunoassay for thyroid stimulating hormone has been perfected and the results used to demonstrate the effectiveness of thyrotropin releasing factor in patients with pituitary dwarfism.

B. Studies of adrenal function with special reference to biogenetic and physiocontrol mechanisms and to the hormonal aspects of hypertension. Control of steroid biosynthesis.

Prostaglandins (PGE_1, $PGF_{2\alpha}$) are released from the adrenal gland upon stimulation by ACTH according to reports by Shaw and Ramwell. In an attempt

to define their effects on adrenal steroidogenesis, prostaglandins (PGE_1, PGE_2, PGA, and $PGF_2\alpha$) have been infused into an isolated adrenal (Hilton pouch) preparation from a previously hypophysectomized dog. The timed, adrenal venous effluent is being analyzed for steroids in the biogenetic pathways leading to cortisol and to aldosterone.

GPA 2282, (3 methyl-2-(3-pyridyl) indole) (Geigy) has been shown in animal preparations to block the production of aldosterone and to promote an increase in the production of 11 desoxycorticosterone. Thus, it was considered a candidate for trial in the medical treatment of aldosteronism. The results to date show that (1) in a patient with primary aldosteronism treated for 44 days with the drug, aldosterone production fell to one-half its initial values, but not to normal. There was no effect on blood pressure during this period and no changes in sodium, potassium or magnesium balance. (2) In a second patient with the secondary aldosteronism of Bartter's syndrome, there was no change in any of these variables, including aldosterone, on treatment with this drug.

Studies with an inhibitor (Aminoglutethimide) that blocks the conversion of cholesterol to $\Delta 5$ pregnenolone in the biogenetic pathway were performed in two sisters with hypertension typical of that of "steroid"origin but without demonstrable increased steroid production. Eight days of treatment produced a slight fall in the aldosterone secretion rates, but effected no change in the blood pressure, serum potassium or sodium and potassium balances. Studies with metopirone (SU-4885) (an 11βhydroxylase inhibitor) in these same patients caused a fall in aldosterone secretion rate but an increase in corticosterone secretion rate. When ACTH-suppressive doses of dexamethasone were imposed on this regimen, the direction of the production rates of these two steroids reversed: aldosterone secretion rose and corticosterone secretion fell. Since plasma renin activity was extremely low at all times throughout this study, a factor other than ACTH or the renin-angiotensin system seems to be effective in stimulating steroidogenesis at a step in the pathway below corticosterone.

Hormonal aspects of hypertension.

The role of steroids in the pathogenisis of hypertension has been studied in several groups of patients, namely:

(a) Patients with hypertension, hypokalemia, and aldosteronism.

Twenty-one patients with hypertension were subjected to a diagnostic test for primary aldosteronism. This consists of measuring blood pressure, serum potassium, aldosterone secretion rates and plasma renin activities on low (9 mEq a day) and high (249 mEq a day) sodium intakes. A fall in serum potassium below 3.5 mEq/l on the high salt intake with continued high aldosterone and suppressed renin are the criteria for primary aldosteronism. Of the twenty-one patients tested, only two had primary aldosteronism, one of which had one hyperplastic adrenal removed and the other is being treated medically to reduce her hypertension.

Various factors and disease states were involved in the production of the hypertension, hypokalemia, and aldosteronism or high plasma renin in the other

patients. These include contraceptives, hypothyroidism, and polycystic kidneys. Four of the patients studied are considered to have essential hypertension.

Four patients with proven hyperparathyroidism and hypertension have been studied to evaluate their aldosterone-renin-angiotensin system. Of these one was proven to have primary aldosteronism, one has normal aldosterone secretion and plasma renin, one has high aldosterone secretion with normal plasma renin, and the forth has high aldosterone with high renin. Thus, the status of the aldosterone-renin-angiotensin system in the hypertension of hyperparathyroidism showed no consistent pattern.

The circadian aspects of blood pressure are being studied in normal subjects and in patients with hypertension, on and off medication. All multiple daily blood pressure measurements are being subjected to computer analysis. Preliminary data support the concept that there is a circadian rhythm in the blood pressure of all patients with hypertension, with an early morning low and a peak between 4 and 8 pm. To further clarify this concept, all new patients with hypertension are attached to a non-invasive blood pressure measuring instrument which records blood pressure every thirty minutes for a period of 48 to 72 hours. These data, and self-measurement data taken at home at frequent intervals are being analyzed for periodic parameters, to wit, phase, amplitude and period.

An even more intensive study in circadian periodicity is being conducted to evaluate the circadian aspects of other variables and to attempt to establish causal relationships. Patients with hypertension are studied on metabolic balance for thirty days during which time measurements made every three hours include blood pressure, mood changes, salivary Na, K and steroids, and urinary Na, K, Ca, P, Mg, 17-hydroxysteroids, 17-ketosteroids and aldosterone. Aldosterone secretion rate and plasma renin activity were also measured as periodic functions. To elicit phase shifts in these variables, the patient's wake-sleep pattern is reversed in the middle of the study.

(b) Patients <u>without</u> hypertension, but with hypokalemia, aldosteronism and elevated plasma renin activity (Bartter's syndrome).

In patients with Bartter's syndrome, two studies were performed to test the autonomy of the aldosterone and renin productions and to attempt to define the pathogenesis of this syndrome.

Three patients were given a Na load of 400 mEq a day and, at a separate time, 100 grams of albumin, to expand their ECF and IVF volumes, respectively. Although the ASR decreased, it did not achieve normal values, and the patients were hyporesponsive to angiotensin infusions at this time. When a vasopressor agent was given for a prolonged period, the blood pressure increased and the plasma renin activity fell, but not to normal values. Thus, these studies provide evidence against a major role for Na-depletion or hypovolemia in the pathogenesis of this syndrome. They also support the concept of unexplained vascular hyporesponsiveness to angiotensin II infusions as a continuing stimulus to excessive renin and aldosterone production.

(c) Patients with aldosteronism, high plasma renin activities and subacute

necrotizing encephalopathy.

Elevated renin and aldosterone secretion rates have been found in four patients with the syndrome of "ophthalmoplegia plus" (elevated cerebrospinal fluid protein, progressive external ophthalmoplegia and pigmentary degeneration of the retina). All patients manifested hypokalemia: two had hypomagnesemia as well. Aldactone given to one patient corrected the hypokalemia but had no effect on the hypomagnesemia. Autopsy in one of the patients revealed subacute necrotizing encephalopathy, hyperplasia of the juxtaglomerular apparatus and nodular hyperplasia of the zona glomerulosa of the adrenal gland.

C. Studies of renal function.

Factors controlling tubular reabsorption of sodium were pursued in physiological and clinical studies. Alpha adrenergic stimuli were shown to increase reabsorption of sodium proximally, as reflected in a decrease of urinary volume and free water clearance during water diuresis. Beta-adrenergic stimulation had the opposite effects; these were thought to be mediated by cyclic AMP, since dibutyryl cyclic AMP has effects very similar to those of beta-adrenergic stimulation.

Studies of the effects of prostaglandins on sodium transport have shown an increase in sodium excretion in response to several classes of these agents, an effect mediated at least partially by a decrease in proximal tubular sodium reabsorption, as suggested by increases in urinary volume, free water clearance and sodium excretion in water-loaded dogs. Studies of various other indices of renal function, including urinary excretion of cyclic AMP, are underway.

The effect of vena cava constriction on sodium excretion and on the renal blood flow was studied by clearance techniques and by kinetics of radioactive Xenon given into the renal artery. Whereas sodium excretion decreased markedly with constriction, there was no statistically significant shift of renal blood flow from superficial to deeper glomeruli. The natriuresis of fasting and its reversal by carbohydrate was further studied. Whereas it was confirmed that organic aciduria (and anion "drag") always accompanied the phenomenon, early results suggest that reversal by carbohydrate may take place locally in renal cells.

In fourteen patients with idiopathic edema it was shown that all had subnormal plasma volume and most had hypo-albuminemia as well. The proximate cause was a low rate of synthesis or a high rate of destruction of albumin or both. Physiologic consequences of the low plasma volume, resulting in edema, were shown to include increased secretion of catecholamines, increased plasma renin with exaggerated response to the upright posture, and increased aldosterone secretion. With aldosterone antagonists, despite very high sodium intake, the patients lost much more weight and sodium ($p < 0.01$ for both) than normals.

D. Studies of the properties of hormonal and other peptides.

The complete primary structure of bovine parathyroid hormone was elucidated for the first time. The hormone was shown to consist of 84 amino acids in a single non-cross-linked chain with N-terminal alanine and carboxyl-terminal

glutamine. The secondary structure of bovine parathyroid hormone was investigated by circular dichroism, optical rotatory dispersion and infrared spectroscopy: tertiary interactions of the hormone were assessed by the fluorescence and near-ultraviolet circular dichroism of the aromatic amino acids. From the circular dichroism in aqueous solution, in 6M guanidine, and in 2 chloroethanol, it was found that bovine parathyroid hormone contains 10 to 15% alpha helical structure in aqueous solution, reduced to random coil in guanidine, and increased to 50% helix in 2 chloroethanol. Studies of tertiary structure suggest a conformational association between tyrosine and tryptophan residues in the molecule.

Similar studies of secondary and tertiary structure were applied to salmon thyrocalcitonin. They revealed a peptide chain dominantly in random coil, with one to two turns of helix in aqueous solution, changing to random coil in 6M guanidine, and to 25 to 30% helix in 2 chloroethanol. Studies of the tertiary structure of salmon calcitonin suggest that the tyrosine residue has restricted rotational freedom, but is completely accessible to solvent. Since these properties of salmon calcitonin closely resemble those of bovine thyrocalcitonin, porcine thyrocalcitonin, and human thyrocalcitonin, the greatly enhanced activity of salmon as opposed to the other calcitonins probably resides in the difference in amino acid sequence rather than in the conformation of the molecule as a whole.

In studies of the immunoreactivity of porcine thyrocalcitonin, it was found that antibody to the whole molecule showed no cross-reactivity with the end terminal 9 amino acids of the molecule, but a high degree of immunological reactivity with the carboxyl terminal 23 amino acids. In view of the marked differences in the structure of this 10-to-32-amino acid peptide and the corresponding region of human or salmon thyrocalcitonin, it is not surprising that there was little immunologic cross reactivity of porcine antibody with human or salmon thyrocalcitonin.

Studies were begun on the primary, secondary and tertiary structure of a carboxyl terminal alanine peptide derived from very-low-density-lipoprotein (VLDL) particle. Fragments of the peptide obtained by enzymatic and chemical cleavage were analyzed for amino acid sequence, and a provisional structure of the peptide was established. In studies of the secondary and tertiary structure, it was found that the peptide has a 10 to 15% helical structure in aqueous solution, changed to random coil structure in 6 M guanidine, and increasing to 80 to 90% of alpha helix in 2 chloroethanol. Studies of tertiary structure suggest that the tyrosine and tryptophan molecules are in close proximity in the sequence. It may be that the structure of the VLDL-protein-peptide attains considerably more helical structure when it interacts with its normal lipid moiety.

Serial No. NHLI-223(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the control of calcium absorption: Effect of sodium chloride loading and depletion.

Previous Serial Number: None.

Principal Investigators: Transbol, I., M.D., Meyer, W.J., M.D. and Middler, S., M.D.

Project Description:

Objectives:

The study was designed to determine the effect of salt loading and salt depletion on urinary and serum calcium, phosphorus, and magnesium and the gastrointestinal absorption of calcium.

Methods Employed:

Normal volunteers and patients being evaluated for primary aldosteronism are admitted to the Clinical Center and placed on a strict metabolic balance of sodium, potassium, calcium, phosphorus and magnesium. The total calcium intake for each day of the study is 800 mg. Two sodium intakes of 12 days each were utilized; 249 mEq and 9 mEq. Blood, urine and stool are analyzed for calcium, phosphorus, magnesium, sodium and potassium. Calcium absorption from the gastrointestinal tract is determined by arm counting after the administration of an oral dose of radioactive calcium.

Major Findings:

Preliminary results on 7 patients and 2 normals are available. The table shows the average calcium excretion for the last four days on each salt intake and the calcium absorption for each salt intake.

Patient	Diagnosis	9 mEq diet 24 hr. urine Ca mg.	% abs.	240 mEq Diet 24 hr. urine Ca mg.	% abs.
G.P.	Normal	82	39	168	48
R.W.	Normal	36	31	64	30
J.K.	Periodic par.	105	43	131	50
D.B.	Nephrocal.	114	37	321	44
E.M.	Hypertension	141	38	120	52
H.Z.	Hypokalemia Myxedema	54	40	42	43
J.E.	J.G. Hyper.	52	43	42	54
W.G.	Hypoparathy.	323	57	355	68
M.G.	Primary aldo.	---	45	---	58

In the seven studies where balance data are now complete there is no effect on calcium balance.

Publications:

None.

Serial No. NHLI-224(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evaluation of secondary hypothyroidism with synthetic thyrotropin releasing factor (TRF).

Previous Serial Number: None.

Principal Investigators: Meyer, W.J., M.D., and Bartter, F.C., M.D.

Other Investigators: Smith, H.

Cooperating Units: Guillemin, R., M.D., Salk Institute, San Diego, California, and Murad, F., M.D., University of Virginia, Charlottesville.

Project Description:

Objectives:

Synthetic TRF has been shown to release TSH from the anterior pituitary in normal human beings. In those patients with secondary hypothyroidism, hypothyroid patients with TSH-responsive thyroid glands, TRF may be a useful tool to further understanding of their disease and possibly for treatment of their disease.

Methods Employed:

Patients with secondary hypothyroidism are admitted to the Clinical Center three weeks after stopping exogenous thyroid medication. The patients are given intravenously 0.75 mg of synthetic TRF made by Dr. Roger Guillemin and Dr. Roger Burgus. RAI uptake is measured the week before receiving the TRF and the day after. PBI and thyroxine levels are measured the day before and for two days after receiving TRF. At the time TRF is given, plasma TSH is measured at the following times: control, zero time, 10 min., 20 min., 30 min., 40 min., 60 min., 90 min., and 120 min. TSH values are determined by radioimmunoassay. For the three-hour period during which the TRF experiment is taking place a urine is collected for cyclic AMP content and compared to a control period urine. Normal volunteers have also been given TRF in order to establish a basis of comparison for the patients.

Major Findings:

Ten patients with secondary hypothyroidism and five normals (one twice) have now received TRF. There have been no side effects noted except a previously reported transient nausea period immediately after receiving TRF in about 50% of the subjects. The following data are now available:

Patient	Diagnosis	RAI 24 hr. bef.,aft.	thyroxine bef., aft.	Baseline TSH uU/ml	Peak TSH	Peak Time min.
B.C.	Normal	---	3.8, 4.0			
G.P.	Normal	---	3.9, 4.4			
J.S.	Normal	---	3.1, 4.0			
R.W.	Normal	---	3.4, 4.3	22.1	36.8	30
R.W.	Normal	---	3.8, 4.1			
V.S.	Normal	---	3.3, 4.2	36.5	110.8	20
L.M.	Hypothalamic	7.5,6.1	2.9, 2.8	100.	320	30
N.S.	Hypothyroid	7.1,15.2	1.8, 2.4	56.6	91.7	20
C.M.	Hypopituitary	6.0,7.5	4.0, 3.3	33.0	125.2	40
S.Z.	Myxedema	9.5,12.2	1.9, 1.9	132.0	197.1	20
S.Z.	Myxedema	---	1.3, 1.1	135.6	310.4	30
E.B.	Hypopituitary	0.8,0.0	1.4, 1.1	18.5	27.1	20
K.U.	(Pseudohypo-	21.4,19.6	2.4, 2.3	50.5	108	30
E.M.	parathyroid)	29.9,23.0	2.5,2.2	58.4	180	10
J.L.	Hypopituitary	2.7, 4.2	2.4, 4.3	20	120	40
J.B.	Low T-4			5	14.6	10
H.J.	Scleroderma	5.9, 7.8	2.0, 2.4			

Urinary cyclic AMP measured by Dr. Ferid Murad showed no difference between control and TRF periods in nine subjects. Synthetic TRF response followed by plasma TSH seems to be a good method of gathering further information about the etiology of secondary hypothyroidism.

Publications:

None.

Serial No. NHLI-225(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Pathophysiologic studies in hypertension

Previous Serial Number: 107(c)

Principal Investigators: Meyer, W.J., M.D., Bartter, F.C., M.D. and Delea, C.S.

Project Description:

Objectives:

(1) To define the roles of the adrenal steroids and of the renin-angiotensin system in the etiology of hypertension.

(2) To investigate the interrelationships of the adrenal glands and the parathyroid glands in patients with hyperparathyroidism and hypertension.

(3) To demonstrate the presence or absence of circadian rhythms in patients with hypertension and to attempt to establish causal relationships among the variables measured.

(4) To continue to investigate the role of the adrenal steroids in patients with Bartter's syndrome (juxtaglomerular hyperplasia, hypokalemia, hyperaldosteronism with normal blood pressure.

Methods Employed:

Fourteen patients with hypokalemia and/or hypertension were studied on two different sodium diets. Blood pressure, serum K, ASR and PRA were measured. In one set of patients urinary calcium and gastrointestinal absorption of calcium were measured also. In the circadian study, blood pressure, mood changes, salivary Na, K and steroids, ASR, PRA, urinary Na, K, Cl, Ca, P, 17 OHCS, 17 KS and aldosterone were measured every three hours.

Major Findings:

(1) Patients with hypertension and/or hypokalemia were studied to evaluate the role of the adrenal steroids and the renin-angiotensin systems in the etiology of their hypertension. The results are as follows: See Table I.

Table I

Patients with Hypertension Or Possible Diagnosis of Primary Aldosteronism (alone)

Patient	Na Intake	B.P.	Serum K	Aldo	Renin S U	Serum mg	
S.Z.	9	150/70	3.8-4.5 (33 d.)	858	1491/1715		Hypothyroidism, diabetes
	240		4.1-4.1	1030	/455		
J.S.	9	190/110	3.7-3.7	389	100/228		
	240	170/110 (aldomet)	3.5-3.5	365	100/ 100		*Primary aldosteronism
	59	194/130	3.8-3.0	794	433/697	1.65-1.90	
W.K.	109	145/90	4.0-4.3	679		1.50-1.55	Hyperthyroidism ?
	249			335		1.65-1.34	
J.H.	9	132/84	3.8-3.4	712	215/589		Polycystic kidneys
	240	130/94	3.3-3.2	154	100/264		
A.F.	9		3.6-3.4	1209	958/1438		
	109	148/106	3.8-3.6	267	441/363		Normal aldo, + renin
	240		4.0-3.3	120			
C.G.	9		3.5-4.0	679	-----		Norinyl 2 mg/d. - 20 d/mo. ?
	240	170/120	4.2-3.5	173	464/3305		ACTH for 6 mo. q 4 hrs.
R.B.	240	150/110	4.3-4.2	321	162/954		? Essential
M.A.	9	160.110	3.7-3.5	---	2061/---		Primary aldosteronism
	240		3.6-3.7	770	--------		Rt adrex and nephrex at 18
D.A.	9	180/130	3.4-3.9	2008	808/1750		On apresaline and
	240	168/112		3200			aldomet ? Essential
	109	198/140	3.8-3.4	286	-------		On aldomet alone not enough data
P.P.	9	140/90		428	182/997		Off contraceptives, pressure returns
	240	130/78		65	139/149		to normal
		9 + Hg	3.3-4.2	1770	1649/1208		
J.K.	240		5.2-3.6	1153	183/121		? Periodic paralysis
	9		4.0-4.5	508	950.1148		JG renal biopsy
J.K.	9	140/80	3.9-3.6	455	496/655	1.50-1.60	Periodic paralysis
	249		4.0-3.3	554	250/97	1.50-1.50	
J.E.	9	120/80	2.1-2.4	531	571/1479	1.45-1.30	JG hyperplasia
	240	120/90	3.7-2.9	124	315/451		
				302	359/1454	0.8-1.25	Unresponsive to angio inf. renal bx.

Proposed Course of Study:

1. To continue to screen patients diagnostically for primary aldosteronism.

2. To measure blood pressure every 30 minutes for 48-72 hours and subject it to cosinor analysis for period, phase and amplitude.

3. To have patients perform self-measurements of blood pressure at frequent hourly intervals at home for long periods of time, e.g., 3 months.

4. To establish the best times of day for medication to control blood pressure in the light of circadian blood pressure measurements.

Major findings:

(2) Four patients with concurrent hypertension and hyperparathyroidism, hypercalciuria and nephrolithiasis were studied to evaluate their adrenal-renal systems. Three other patients with hypercalciuria and stones were evaluated in the same way. The results are as follows: See Table II.

Table II

Patient	Na Intake	B.P.	Aldo	Renin	Stones	Ca	Operation
(pts. with hyperparathyroidism)							
T.W.	9	150/100	504	533/522	X	200	PTX-hyperplasia
	249	134/98	121	187/345			
E.B.	9	160/110	1795	358/1371	X	200-250	PTX-adenoma
	249	180/100	776	123/635			
W.G.	9		788	/2424	X		PTX
	249		1664	323/780			
S.D.	9		--	0/0	X	250-350	Adx-hyperplasia
	249		107				Normocalcemic
	109		240	290/			hyperparathyr.
	249		512				tests - for
	9		---	273/			
	249		1115				
	250		257	231/353			Post-adx.
(pts. with hypercalciuria, nephrolithiasis)							
D.B.	9	120/80	423	568/	X	over 300	PT test - neg.
	249	134/100	166	331/			
P.M.	9		611	839/2614	No	over 300	PT test - neg.
	240		914	/1884			
M.M.	9	120/80	?	?	X	250	Normal PT
	249	136/84					

Of the first four, S.D. and T.W. had suppressed plasma renin activities. S.D. also had pronounced hypokalemia. On operation she was found to have adrenal hyperplasia; one gland was removed, the other biopsied. Although she is thought to have normocalcemic hyperparathyroidism, she has not been subjected to another operation because of her cardiac status; her renal stones and hypercalciuria are controlled with cellulose phosphate.

In the light of the extremely high plasma renin activities, W.G. was thought to have the syndrome of juxtaglomerular hyperplasia. Accordingly, he was given an intravenous infusion of angiotensin and norepinephrine. His diastolic pressure failed to rise 20 mm of Hg at an infusion rate several times that to which a normal person responds. A renal biopsy was performed to define the lesion. The results are pending.

Major Findings:

(3) The circadian aspects of blood pressure are being studied in normal subjects and in patients with hypertension, on and off medication. All multiple daily blood pressure measurements are being subjected to computer analysis. Preliminary data supports the concept that there is a circadian rhythm in the blood pressure of all patients with hypertension, with an early morning low and a peak between 4 and 8 p.m. To further clarify this concept, all new patients with hypertension are attached to a non-invasive blood pressure measuring instrument which records blood pressure every thirty minutes for a period of 48 to 72 hours. These data, and self-measurement data taken at home at frequent intervals are being analyzed for periodic parameters, to wit, phase, amplitude and period.

Major Findings:

(4) In patients with Bartter's syndrome, two studies were performed to test the autonomy of the aldosterone and renin productions and to attempt to define the pathogenesis of this syndrome.

Three patients were given a Na load of 400 mEq and, at a separate time, 100 grams of albumin to expand their ECF and IVF volumes respectively. Although the ASR decreased, it did not achieve normal values and the patients were hyporesponsive to angiotensin infusions at this time. When a vasopressor agent was given for a prolonged period, the blood pressure increased and the plasma renin activity fell, but not to normal values. In the light of these results, it was concluded that these studies provide evidence against a major role for the Na depletion or hypovolemia in the pathogenesis of this syndrome. They also support the concept of unexplained vascular hyporesponsiveness to angiotensin II infusions as a continuing stimulus to excessive renin and aldosterone production.

Proposed Course of Study:

Since K loss was a constant problem in these patients, they were given triamterine throughout the course of the Na-loading. This regimen must be tried in normal subjects to assess the effect of it on the ASR and PRA's. In addition, to obtain further information on the vascular resistance to angiotensin, this infusion must be given to these patients at a time when the blood pressure has been maintained at high values with vasopressor agents for prolonged periods of time. Since many of these patients exhibit hypomagnesemia, the state of their magnesium metabolism should be investigated in relation to their adrenal status.

Publications:

1. Halberg, F., Nelson, W., Doe, R., Bartter, F.C. and Reinberg, A.: Chronobiologie. Journal of European Toxicology 6: 311, 1969.

2. George, J.M., Wright, L, Bell, N.H., Bartter, F.C. and Brown, R.: The syndrome of primary aldosteronism. Am. J. Med. 48: 343, 1970.

3. Bartter, F.C. and Mills, I.H.: Further evidence that renin is not the intrarenal natriuretic hormone. J. Endocrinology 48: xii-xiii, 1970.

4. Bartter, F.C.: So-called Bartter's syndrome. New England J. Med. 281: 1483, 1969.

5. Bartter, F.C., Bravo, E.L., and Delea, C.S.: The role of steroids in hypertension. In James, V.H.T. (Ed.): Proceedings of the Third International Congress in Hormonal Steroids, Sept. 7-12, 1970. Amsterdam, Excerpta Medica Foundation, 1971.

Serial No. NHLI-226
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The effect of prostaglandins upon cortisol and aldosterone secretion of hypophysectomized-nephrectomized dogs.

Previous Serial Number: None

Principal Investigators: Meyer, W.J., M.D., and Bartter, F.C., M.D.

Other Investigators: Casper, A.G.T., Kelly, G. and Tate, J.

Project Description:

Objectives:

The importance of prostaglandins in the function of the adrenal cortex is unknown. Shaw and Ramwell have shown that they are released from the adrenal gland upon stimulation with ACTH. Flack, working in the same laboratory, has shown that PGE_1, PGE_2, and $PGF_{2\alpha}$ increase steroidogenesis in the continuous superfusion of the rat adrenal, but Funder demonstrated variable effects on steroidogenesis in the autotransplanted adrenal gland of the sodium-depleted sheep. The purpose of this study is to try to further clarify the role of prostaglandins in adrenal cortical function.

Methods Employed:

Normal adult male mongrel dogs, weighing between 20 and 30 kilograms, are anesthetized by intravenous administration of sodium pentobarbital. The hypophysis is removed by the transbuccal approach. Then both kidneys are removed and the adrenals are prepared for perfusion by the technique of Hilton et al. Donor hypophysectomized-nephrectomized dogs were bled from the femoral artery before the day of the above preparation. The blood is used to support blood pressure and is given at least volume for volume of that lost. When necessary, norepinephrine is also used to support blood pressure. Thirty minutes after the preparation is completed and the blood pressure is stable, infusion of saline is begun into the arterial side of the preparation at 0.573 cc/min. The entire venous output of the adrenal pouch is collected. The saline is run for three five-minute consecutive periods; then a solution containing a thirty micromolar solution of a prostaglandin in saline is substituted. The prostaglandin-containing solution is chosen to produce an adrenal arterial concentration between one and two micromolar prostaglandin. The blood flow rate through the glands usually runs between five and fifteen ml/min. depending on the individual preparation. The flow rate can be held within one or two ml./min. for each preparation. The prostaglandin is infused for twelve consecutive five-minute periods or one hour. Toward the end of the experiment the entire venous

flow is obstructed and an arterial sample is taken directly from the adrenal pouch to determine the actual prostaglandin concentration. The adrenal venous blood will be analyzed for cortisol by the Porter-Silber method and aldosterone by the radioimmunoassay. All of the prostaglandin will be tested twice in this preparation.

Major Findings:

To date the following prostaglandins have been used in the preparation: PGE_1, PGE_2, PGA_1, and $PGF_{2\alpha}$. No analysis of the adrenal venous blood has been completed.

Publications:

None.

Serial No. NHLI-227
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the etiology of the natriuresis of fasting

Previous Serial Number: None.

Principal Investigators: Meyer, W.J., M.D.

Other Investigators: Kelly, G. and Tate, J.

Project Description:

Objectives:

In man after a twelve-hour fast, a natriuresis begins. This natriuresis persists until refeeding and is greater than that on a 9 mEq sodium diet. The natriuresis is reversed within thirty minutes after giving I.V. glucose and within one hour of oral glucose. The purpose of this study is to document these phenomena in the dog and to demonstrate their intrarenal origin.

Methods Employed:

Three adult female dogs with episiotomies were placed on a 60 mEq sodium diet and strict urinary collections. They were then fasted for seven days followed by refeeding with the 60 mEq sodium diet. The dogs were given by stomach tube at least 1000 ml of water each day during the entire period. Total urinary excretion was determined for each day.

Another group of dogs was fasted for four days receiving at least 1000 ml of water each day. On the morning of the fifth day they were given 1500 ml of water over one hour; thirty minutes later they were placed under pentobarbital general anesthesia. Then a mannitol diuresis was started with a priming dose of 12 grams of mannitol followed by a 5 cc/minute infusion of 2.5% mannitol in distilled water. In the left renal artery a glucose solution in 2.5% mannitol was begun at 0.2 cc/minute. Clearances of inulin, para-aminohippurate and sodium were determined for each kidney over each thirty-minute period. The total experiment consisted of three control periods and six experimental periods. Two concentrations of glucose have been tried: five percent and 0.5 percent.

Major Findings:

The data for the urinary excretion of sodium in the three fasted and refed dogs are tabulated for the last two days of fasting and the first two days of refeeding. They demonstrate that refeeding a 60 mEq sodium diet did not reverse the natriuresis of fasting, but sodium retention can be demonstrated.

Dogs	Day of Fasting 6th	7th	Day of refeeding 1st	2nd	3rd
Jo	4.2	3.1	44.0	31.3	71.2
Mary	3.9	3.3	46.8	35.5	50.6
Gloria	2.9	1.3	1.0	12.5	79.5

The data on the clearances of the anesthetized dog experiments are not completed.

Publications:

None.

Serial No. NHLI-228(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The use of 17∝estradiol in the inhibition of the effect of D-Penicillamine on collagen solubility in skin.

Previous Serial Number: None.

Principal Investigators: Meyer, W.J., M.D., Pak, C.Y.C., M.D., and Bartter, F.C., M.D.

Cooperating Units: Henneman, D., M.D., Ortho Research Laboratories, Raritan, N.J.

Project Description:

Objectives:

D-Penicillamine is known to reduce the tensile strength of skin and increase its neutral salt-soluble collagen. In the rat osteoporosis and bone deformities occur if large doses of penicillamine are administered. Patients with cystinuria are one group of patients which must have D-penicillamine in order to stay symptom-free, and many of these patients also have demonstrable skin side effects of penicillamine. 17∝estradiol has been shown in rats to protect against the penicillamine-induced skin and bone abnormalities without showing any effect on the menstrual cycle. Therefore this particular estrogen was selected for trials in humans, male and female, taking penicillamine for treatment of cystinuria with demonstrable skin collagen changes noted on biopsy.

Methods Employed:

Patients taking D-Penicillamine for cystinuria are admitted to the Clinical Center for a period of two months. They are placed on a strict metabolic balance of sodium, potassium, calcium, phosphorus, and magnesium, monitoring urine, stool and blood. In addition to the above, urinary cyclic AMP, free and total hydroproline, creatinine excretion, and protein are also followed daily. The hospitalization is divided into three periods: twelve day control, twenty day of 10-15 mg/kg/day subcutaneous 17∝estradiol, twelve day post drug. During each period the following will be checked at least once: clotting factors, serum copper, ceruloplasmin, plasma parathyroid hormone, thyrocalcitonin, renin activity and substrate, serum insulin, serum growth hormone, thyroid binding globulin with thyroxine, plasma cortisol, testosterone binding protein, cuff test for capillary fragility and skin content of soluble collagen. With the above tests the spectrum of estrogen-like effect of 17∝estradiol can be evaluated as well as its inhibitory effect of the deleterious action of D-penicillamine on skin.

Major Findings:

To date three patients have begun on the above protocol. There are no data available at this time.

Publications:

None.

Serial No. NHLI-229(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The use of 3-methyl-2-(3-pyridyl)-indole methanesulfonate (GPA 2282) in the treatment of hyperaldosteronism.

Previous Serial Number: None.

Principal Investigators: Meyer, W.J., M.D. and Bartter, F.C., M.D.

Cooperating Units: Socolow, E., M.D., Geigy Pharmaceuticals, Ardsley, N.Y.

Project Description:

Objectives:

GPA 2282 has previously been shown in vivo in the dog and in vitro in the adrenal cortex of the dog, cow, and human being to block the production of aldosterone by Doctors Bartter and Burwell. At the time of this blockade there was a rise in 11-desoxycorticosterone production. Human studies done elsewhere with normals and patients with primary aldosteronism have been inconsistent.

Methods Employed:

Three patients with hyperaldosteronism were selected to be admitted to the hospital for evaluation of the GPA 2282 under rigorous metabolic regimen, receiving constant dietary intake and collecting all urine and stools for complete balance measurements of sodium, potassium, calcium and magnesium. After an initial eight-day control period the GPA 2282 was begun at 250 mg every 6 hours p.o. for a period up to 44 days. In addition to the balance data aldosterone secretions and supine and upright renins were drawn each week of the study. The following parameters were followed in order to check for drug toxicity: CBC, platelet count, bleeding time, clotting time, prothrombin time, partial prothrombin time, stool guaiac, creatinine clearance, SGOT, bilirubin total and direct, alkaline phosphatase, uric acid, sedimentation rate and urinalysis.

Major Findings:

Two patients have now completed the study: a 40-year-old white female with juxtaglomerular hyperplasia, hypokalemia, aldosteronism, elevated plasma renin activity without hypertension; and a 42-year-old white female with bilateral adrenal hyperplasia with primary hyperaldosteronism. No signs of toxicity were noted; however, both patients complained that the medication "burned" each time they took it. The medication had no effect on the patients' blood

pressure. The complete balance data is not available but the GPA 2282 had no effect on the serum and urinary values of sodium, potassium, calcium and magnesium. The renin values have not been determined. The aldosterone secretion values that follow show only some beneficial effect in the patient with primary aldosteronism.

Day:	Control	5th	8th	14th	16th	22nd	23rd	32nd	41st
JG Hyperplasia	215		745		414	540			
Primary Aldo.	794	834		576			306	398	317

Publications:

None.

Serial No. NHLI-230(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The circadian urinary excretion of ketosteroids in patients with the adrenogenital syndrome.

Previous Serial Number: None.

Principal Investigators: Meyer, W.J., M.D. and Bartter, F.C., M.D.

Other Investigators: Diller, E.

Project Description:

Objectives:

Because patients with the adrenogenital syndrome produce inadequate amounts of cortisol, they produce large amounts of ACTH and ketosteroids. This study is designed to determine if there is a circadian variation of ketosteroids in these patients and what the characteristics of that variation are with and without therapy.

Methods Employed:

Patients with both salt-losing and non-salt-losing virilizing adrenal hyperplasia are admitted one month after their last DOC pivalate injection to the Clinical Center. The patients begin a fluid intake in excess of 2000 cc/m^2/day divided equally into four hour periods. Vital signs are taken every four hours. During four days while they are still taking cortisol replacement and during one week without replacement, they collect urines in four hour periods. The patients are then restarted on therapy and discharged. The urines are analyzed for ketosteroids by the modified Zimmerman method.

Major Findings:

Eight patients have completed the above protocol. Four of the patients have the salt-losing form of the syndrome. No urinary ketosteroid analyses are available yet.

Publications:

None.

Serial No. NHLI-231(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Calcium metabolism in cystic fibrosis.

Previous Serial Number: NHLI-127(c)

Principal Investigators: Simopoulos, A.P., M.D., Pak, C.Y.C., M.D. and Bartter, F.C., M.D.

Cooperating Units: Kattwinkel, J., M.D. and di Sant'Agnese, P.A., M.D., Pediatric Metabolism Branch, NIAMD, and Murad, F., M.D., University of Virginia, Charlottesville.

Project Description:

Objectives:

Patients with cystic fibrosis have pancreatic deficiency and fat intolerance. Standard textbooks describe the radiologic findings of the bones as being consistent with "osteoporosis". In order to evaluate bone metabolism in this disorder the following study was set up.

Methods Employed:

Five patients with cystic fibrosis, 14-23 years of age, were studied under strict metabolic conditions on a constant Ca and P intake, but on 9, 109, and 249 mEq of Na/day.

Calcium infusions were performed on a different admission. All patients were put on a liquid diet containing 400 mg of Ca and 890 mg of P per day. Ca infusion (15 mg/Kg) was given on the third day. Ca and P were analyzed every 12 hours. Urinary cyclic AMP (24 hr) was determined daily.

Plasma was collected for TCT and parathyroid hormone determinations before and after infusion (TCT and PTH are to be determined at a later date).

All patients are to be admitted in April, 1971 for calcium absorption studies.

Results		Calcium Balance
Balance Data	A.J.	+117
	V.J.	+19
	V.S.	+11
	B.M.	+14
	B.S.	-27

	Normal	C.F. Pts.	Hyperparathyroid Pt.
% fall in urinary P during the second 12 hr. of calcium infusion	-52±14.5 SD%	VS -9% VJ -20% AJ -9.9% BM -15% BS +34%	-7.7±14.1 SD%
CAMP μmoles/gm	less than 3.8	6.3	5.4±2.2 SD

Major Findings:

The data indicate that a state of hyperparathyroidism is present in patients with cystic fibrosis.

The calcium absorption data ought to indicate if this state of hyperparathyroidism is primary or secondary to malabsorption of Ca by the gastrointestinal tract.

Publications:

1. Simopoulos, A.P., Lapey, A., Boat, T., di Sant'Agnese, P.A., and Bartter, F.C.: The renin-angiotensin-aldosterone system in cystic fibrosis. In print. Pediatric Research, January 1972.

Serial No. NHLI-232(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The relationship of central nervous system degenerative disorders to the adrenal hyper- and hypoplasia. Studies in: I. Subacute necrotizing encephalopathy (SNE) - Bone marrow and electron microscope studies on brain. II. Sudanophilic leukodystrophy and Addison's disease - Brain electron microscopy.

Previous Serial Number: None.

Principal Investigators: Simopoulos, A.P., M.D.

Cooperating Units: Roth, J., M.D., Laboratory of Pathology, NCI.

Project Description:

Objectives:

I. Previous studies in one patient (V.S.) with clinical and biochemical evidence of SNE established the fact that she was dependent on high doses of thiamine HCl, $MgCl_2$ and Spironolactone in order to remain free of ataxia, maintain normal serum potassium levels and develop physically and normally. In addition, the vacuolization of the bone marrow and the presence of the inhibitor were dependent on thiamine and $MgCl_2$. When the parents withheld medications for 7-10 days the patient had a severe relapse, the vacuolization was present in the bone marrow and in the peripheral blood. At autopsy, the same vacuoles were present in the cortex, the midbrain, cerebellum and the spinal cord. On electron microscopic investigation, these vacuoles appear to be in the intramyelinic axons. These vacuoles did not stain with sudan, ORO, PAS, or any of the other stains. Similar vacuoles have been produced in rats with intracerebral injections of ouabain, indicating abnormal membrane transport.

Although this disease is most likely an inherited metabolic disorder of the autosomal recessive type, sections of brain tissue were injected into monkeys to rule out viral disorder. Results so far (6 months) are negative.

II. Another patient with Sudanophilic leukodystrophy and Addison's disease had brain biopsy. Electron microscopic studies are in progress.

Publications:

1. Simopoulos, A.P., Delea, C.S. and Bartter, F.C.: Neurodegenerative disorders and hyperaldosteronism. J. Pediatrics (in print).

Serial No. NHLI-233(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Calcium metabolism in idiopathic hypercalcemia of infancy and childhood. I. The effect of calcium infusion on serum calcium, thyrocalcitonin (TCT) and parathyroid hormone (PTH. II. 24-hour urinary cyclic AMP in two patients with the disease.

Previous Serial Number: None.

Principal Investigators: Simopoulos, A.P., M.D. and Pak, C.Y.C., M.D.

Cooperating Units: Murad, F., M.D., University of Virginia, Charlottesville

Project Description:

Objectives:

The syndrome of idiopathic hypercalcemia of infancy and childhood remains totally unexplained in terms of etiology, natural evolution of the disease process and relationship of CAMP, TCT, and PTH in the patients who are no longer hypercalcemic. The purpose of this study was to evaluate CAMP, TCT and PTH in patients who no longer required treatment and had been normocalcemic on ad libitum Ca intake over a one-year period. We felt that if an abnormality in this parameter were to be found it would be of great help to identify older children in mental institutions who present with the same physical and mental picture, but in whom, unfortunately, serum calcium levels had not been determined when they were infants.

Methods Employed:

I. Calcium infusion, TCT and PTH before and after infusion.

a) observe the response of urinary P, Ca.

b) the level of serum Ca and the rate of its disappearance.

(Since data on oral Ca loading tests indicate decreased rate of serum Ca disappearance).

II. 24-hr. urinary cyclic AMP.

So far two patients with the severe form of the disease have been studied.

1) Pt. G.G., 4-year-old female. No increase in serum Ca during or following Ca infusion.

24-hr. urinary CAMP 1.2 umoles/gm of creatinine/day is normal. (Normal values less than 3.8 umoles/gm of creatinine/day.)

2) Pt. J.G., 8 1/2 year-old male. No increase in serum Ca during or following Ca infusion, yet patient became hypertensive during the third hour of the Ca infusion.

Urinary P did not change, indicating abnormal Ca infusion test.

Results on TCT and PTH are pending.

The third patient, J.K., is to be studied in the future.

Major Findings:

Conclusions will be drawn when all data are available.

Publications:

1. Simopoulos, A.P., Marshall, J.R., Delea, C.S. and Bartter, F.C.: Studies on the deficiency of 21-hydroxylation in patients with congenital adrenal hyperplasia. J. Clin. Endocr. 32: 438-443, March 1971.

Serial No. NHLI-234
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effect of prostaglandins on the dog kidney

Previous Serial Number: None.

Principal Investigator: Gross, J.B., M.D.

Other Investigators: Bartter, F.C., M.D., Casper, A.G.T., Kelly, G. and Tate, J.

Project Description:

Objectives:

The renal effects of the prostaglandins have been partially explored but not fully elucidated. Effects on GFR, renal blood flow, and renal sodium excretion require further investigation, and the mechanism of prostaglandin-induced natriuresis remains unknown. Complete studies of the effects of the agents on urinary 3', 5' AMP excretion do not exist. The present study was undertaken to elucidate more fully the effect of prostaglandins A, E, and F_2 on renal electrolyte excretion, renal blood flow and GFR, PAH extraction, and urinary excretion of 3', 5' AMP.

Methods Employed:

Female dogs weighing 15-28 kg. were anesthetized with pentobarbital, 30 mg/kg. Light anesthesia was maintained throughout the experiment. A mid-line abdominal incision was made, catheters placed in each ureter, and small cannulas were placed in the left renal artery, and both renal veins. Blood pressure was recorded via an intra-arterial cannula in a femoral artery. In more recent experiments, respirations were controlled via a volume-cycled respirator, and arterial pH monitored and maintained in or near the normal range throughout the experiment.

Dogs were divided into two groups, one receiving an initial waterload, followed by a sustaining dextrose-in-water infusion, and the other being water-restricted and given maximal intravenous doses of antidiuretic hormone. Infusions of inulin and sodium para-amino-hippurate were administered to both groups and inulin clearance, PAH extraction, and renal plasma flow were calculated in the standard manner. 10-minute control and experimental urine-collection periods were utilized in both groups of dogs.

After several initial control periods, the drug was infused into the left renal artery at rates ranging from .1 to 2 micrograms/minute. In several ex-

periments, post-experimental control periods were also utilized. In each experiment, the contralateral kidney served as a simultaneous control for the infused kidney.

The following measurements and calculations were made in each experiment - inulin clearance, PAH extraction, osmolar clearance, renal plasma flow, Na^+ excretion, K^+ excretion, free-water clearance ($C \cdot H_2O$) or free-water reabsorption ($Tc\ H_2O$), systolic, diastolic and mean blood pressure. In several experiments, urine was also collected for 3', 5' - AMP determinations.

Major Findings:

The data are still being collected and are insufficient at the present time to allow interpretation.

Publications:

None.

Serial No. NHLI-235(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the control of sodium excretion. I. Mechanisms of sodium retention.

Previous Serial Number: NHLI-125(c)

Principal Investigators: Gill, Jr., J.R., M.D., Delea, C.S., A.B., and Bartter, F.C., M.D.

Cooperating Units: Waldmann, T.H., M.D., Metabolism Branch, NCI

Project Description:

Objectives:

Previous studies have demonstrated that abnormally low rates of synthesis of albumin or abnormally high rates of albumin catabolism can lead to hypoalbuminemia, hypovolemia and sodium and water retention on the basis of increased adrenergic activity and hyperaldosteronism. In these patients the total albumin pool is also reduced and the distribution ratio of extravascular to intravascular albumin is normal. In some patients the ratio of distribution is abnormal. Such patients are now currently under study to determine the physiological basis of the abnormal distribution and its pathological cause.

Methods Employed:

Radioactive albumin is injected intravenously to evaluate the various parameters of albumin metabolism and the results obtained further analyzed in terms of a three-compartment model to determine the rate of egress of albumin from the intravascular compartment as well as its rate of return.

Major Findings:

In one patient (J.C.) with albumin-rich bilateral pleural effusions, this kind of analysis suggested that a delay in return of albumin to the circulation rather than a rapid rate of egress from the circulation was the basis for the accumulation of albumin in the pleural spaces. Total circulating pool of albumin was also decreased by an unexplained increase in the fraction of intravascular albumin catabolized per day. The pathogenesis of the hypercatabolism and the nature of the abnormality of the lymphatics draining the pleural space remain to be identified. The data for the albumin studies are presented in tabular form below.

	31	13	12	21	41	M_1	M_2	M_3
	fraction per day					gm/Kg		
Normal	.379	.319	3.29	.852	.109	1.56	.44	1.84
±	±	±	±	±	±			
S.E.M.	.053	.031	.372	.117	.007			
J.C.	.422	.125	2.76	1.39	.158	1.21	.63	4.05

31 = fraction of intravascular albumin transferred to large slowly turning over extravascular albumin pool, which presumably includes pleural effusions.

13 = fraction of large slowly turning over extravascular pool transferred to intravascular pool.

12= fraction of small rapidly turning over extravascular pool transferred to intravascular pool per day.

21 = fraction of intravascular albumin transferred to small rapidly turning over extravascular pool.

41 = fraction of intravascular albumin catabolized per day.

M_1 = intravascular pool.

M_2 = small extravascular pool which turns over rapidly.

M_3 = large extravascular pool which turns over slowly.

Publications:

1. Gill, Jr., J.R.: Edema. Ann. Review of Medicine 21: 269, 1970.

Serial No. NHLI-236
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the control of renal sodium excretion. II. The role of adrenergic receptors and cyclic nucleotides.

Previous Serial Number: NHLI-126

Principal Investigator: Gill, Jr., J.R., M.D.

Other Investigators: Casper, A.G.T. and staff

Project Description:

Objectives:

Previous studies from this laboratory have provided evidence that beta adrenergic stimulation decreases the proximal tubular reabsorption of sodium but does not apparently limit sodium reabsorption in more distal areas of the nephron (1). As this decrease in sodium reabsorption was not associated with any change in renal hemodynamics, it was presumed to occur through direct effects on cellular transport processes. A possible mode of cellular mediation of beta adrenergic stimulation is the adenyl cyclase system reportedly present in the renal cortex. This enzyme system responds to beta adrenergic stimulation as well as to stimulation of other receptors such as that for parathyroid hormone by the generation of intracellular 3',5' cyclic AMP.

In support of the proposition that generation of intracellular cyclic AMP can decrease sodium transport are our observations that dibutyryl cyclic AMP (and cyclic AMP) decreases the proximal tubular reabsorption of sodium (2). As in the case of beta adrenergic stimulation, the nucleotide does not appear to limit sodium transport in other portions of the nephron, nor does it alter renal hemodynamics.

Considerable published data indicate that a number of physiologic interventions can decrease the reabsorption of sodium by the proximal tubule. In most cases, changes in peritubular capillary oncotic (or hydrostatic) pressure, either determined or presumed on the basis of changes in renal hemodynamics, were associated with the changes in net tubular sodium transport and invoked to explain them. How changes in peritubular capillary oncotic or hydrostatic pressure might lead to changes in net sodium transport by the renal tubule is conjectural. Intracellular cyclic AMP and plasma membrane receptors which can mediate its formation in proximal tubule cells would appear to constitute a mechanism which, when activated, can directly decrease net transport of sodium by these tubule cells.

In light of these important effects of beta adrenergic stimulation on renal function, the effects of alpha adrenergic stimulation on renal function were determined in the present studies.

Methods Employed:

Water diuresis was produced in anesthetized, hypophysectomized, cortisol-treated dogs by infusion of 2.5 percent dextrose. Beta adrenergic blockade of the left kidney was produced by infusion in the left renal artery of propranolol, .17 μg/Kg/min., in normal saline .5 ml/min. When urine flow was steady, clearance measurements were started. After three control periods were obtained, norepinephrine .009 μg/Kg/min. was added to the solution infused in the left renal artery for four periods. Three or four periods were obtained after norepinephrine was stopped. The clearances of inulin and para-aminohippurate were determined. Changes in urine flow (V) per 100 ml glomerular filtration rate (GFR) were assumed to represent changes in proximal tubular sodium reabsorption. Clearance of solute-free water (C_{H_2O}) was taken as an index of sodium reabsorption by the diluting segment of the nephron.

Major Findings:

The addition of norepinephrine to the left renal artery infusate resulted in a significant ($P < .01$) decrease in urine flow and in C_{H_2O} without a change in the clearance of inulin or in sodium excretion in that kidney; urine flow, C_{H_2O}, C_{In} and $U_{Na}V$ were essentially unchanged in the right kidney. The results are summarized in the table below.

		Right kidney		
	C_{In}	V	C_{H_2O}	$U_{Na}V$
	ml/min	ml/100 ml GFR		mEq/min
Control	33 ± 3	7.0 ± .5	5.5 ± .5	6
Norepinephrine (LRA)	32 ± 2	7.0 ± .6	5.5 ± .7	7
Post Control	33 ± 2	6.9 ± .6	5.5 ± .7	7
		Left kidney		
	C_{In}	V	C_{H_2O}	$U_{Na}V$
	ml/min	ml/100 ml GFR		mEq/min
Control	30 ± 2	7.8 ± .7	6.2 ± .7	7
Norepinephrine (LRA)	30 ± 2	6.3 ± .5	5.0 ± .6	6
Post Control	32 ± 2	7.3 ± .6	5.9 ± .8	9

The data provide indirect evidence that norepinephrine increased proximal

but not distal tubular reabsorption of sodium. The decrease in C_{H_2O} was similar in magnitude to the decrease in V, specifically the fraction of delivered sodium reabsorbed in the loop of Henle ($C_{H_2O}/C_{Na} + C_{H_2O}$) = .97) did not increase with norepinephrine (.97). With norepinephrine, the clearance of para-aminohippurate decreased 9 ml/min. in the right kidney and 11 ml/min. in the left kidney: filtration fraction was similar in the two kidneys (.46 right; .48 left). Similar changes in renal hemodynamics in the two kidneys suggest that the changes in sodium reabsorption with norepinephrine which were limited to the left kidney were probably the result of direct tubular effects of the catechol. Thus, alpha adrenergic stimulation which increases tubular sodium reabsorption affects sodium reabsorption in a direction opposite to that of beta adrenergic stimulation. The effects of alpha adrenergic stimulation on sodium transport, like those of beta adrenergic stimulation, appear to be limited to the proximal tubule. As the effects of beta adrenergic stimulation appear to be mediated through the adenyl cyclase system so also may the effects of alpha adrenergic stimulation be mediated by nucleotide. The possibility that guanyl cyclase and cyclic guanosine 3'5' monophosphate mediate alpha adrenergic stimulation is currently under investigation.

Publications:

1. Gill, Jr., J.R. and Casper, A.G.T.: Depression of proximal tubular sodium reabsorption in the dog in response to renal beta adrenergic stimulation by isoproterenol. J. Clin. Invest. 50: 112-118, 1971.

2. Gill, Jr., J.R. and Casper, A.G.T.: Renal effects of adenosine 3',5' cyclic monophosphate and dibutyryl adenosine 3',5' cyclic monophosphate: Evidence for a role for cyclic AMP in the regulation of proximal tubular sodium reabsorption. J. Clin. Invest. 50: June, 1971.

Serial No. NHLI-237(c)
1. Endocrinology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on control of calcium and magnesium excretion: Intestinal absorption and renal excretion of calcium in metabolic acidosis and alkalosis.

Previous Serial Number: NHLI-124(c)

Principal Investigators: Transbol, I., M.D., Gill, Jr., J.R., M.D., Lifschitz, M., M.D., Delea, C.S. and Bartter, F.C., M.D.

Project Description:

Objectives:

To determine the effect of systemic acidosis and alkalosis on the tubular reabsorption of calcium and magnesium and urinary excretion of these ions and on the gastrointestinal absorption of calcium.

Methods Employed:

Acidosis is produced in patients with renal tubular acidosis by discontinuing treatment and in patients with a normal capacity to excrete acid by treatment with ammonium chloride. Alkalosis is produced by giving sodium bicarbonate, 100 mEq per day, as a replacement for sodium chloride, 100 mEq per day. Ultrafiltrable magnesium and calcium as well as total magnesium and calcium are determined. Calcium absorption from the gastrointestinal tract is determined by arm counting after the administration of an oral dose of radioactive calcium.

Major Findings:

Regimen	Serum bicarbonate mEq/L	Gastrointestinal calcium absorption %	Urinary calcium mg/day
None	28 ± 1	38 ± 5	102 ± 21
Alkalosis	35 ± .5	55 ± 8	81 ± 15
Acidosis	20 ± 1	43 ± 4	214 ± 19

Serum calcium and clearance of creatinine were unchanged by the various regimens. The above results suggest that alkalosis increases the intestinal absorption of calcium and probably the tubular reabsorption of calcium. Conversely,

acidosis appears to decrease the tubular reabsorption of calcium; its effect on the intestinal absorption of calcium is uncertain because of possible stimulation of the intestinal absorption of calcium by parathyroid hormone released by the stimulus of renal calcium loss.

Publications:

None.

Serial No. NHLI-238
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. I. Ionic interaction with bone mineral and biologic calcification.

Previous Serial Number: NHLI-113

Principal Investigator: Pak, C.Y.C., M.D.

Other Investigator: Ruskin, B.

Project Description:

Objectives:

To evaluate the physicochemical factors concerned in the interaction of mineral ions with the solid phase of bone, and to examine biological calcification.

Methods Employed:

The kinetics of uptake of ^{45}Ca and ^{32}P by synthetic calcium phosphates and by mineral phase was evaluated. This permitted calculation of the content of calcium and phosphate in the hydration shell of the crystal, and the uptake of calcium and phosphate by the solid phase alone (exclusive of the hydration shell).

The problem of biological calcification is being evaluated by spontaneous precipitation in an artificial medium and by calcification of the organic matrix.

Major Findings:

Bone mineral is not a single phase, but it is composed of a heterogeneous mixture of calcium phosphates. Numerous ionic substitutions and exchanges presumably occur. Previous studies have characterized the physicochemical properties of bone mineral.

First, the uptake of ^{45}Ca by hydroxyapatite or bone powder could be reversed by wash-out into isotope-free solution containing stable calcium. This constituted experimental evidence for isoionic calcium exchange. Second, certain univalent cations (Na, K, Li, Cs) could substitute for calcium at the crystal surface on a one-to-one basis. Larger cations (Tris and TMA) did not participate in such an interaction.

Third, synthetic calcium phosphates with low Ca/P ratio have a greater affinity for Ca (2+) than those with high Ca/P. Fourth, effect of F (-), SO_4 (2-), Mg (2+) and citrate on the exchange of ^{45}Ca and $^{32}PO_4$ with the mineral phase of bone was determined under physiologic conditions. Mg (2+) substituted for Ca (2+) at the hydration shell, while citrate (3-) displaced PO_4 (3-) from both the hydration shell and the crystal surface. These bone-seeking elements thus increased the apparent solubility of bone mineral. In contrast, F (-) markedly reduced the concentration of Ca (2+) and PO_4(3-) in both the hydration shell and the ambient solution, probably by inducing the formation of a Ca F_2 boundary layer at the crystal surface. There was a net uptake of Ca (2+) and PO_4(3-) by the solid phase in the presence of Mg (2+) and F (-), but not in citrate solutions. Sulfate (2-) did not significantly affect the solubility, dissolution or growth of bone mineral.

These studies are now extended to the problem of biological calcification. Initially, calcification in an artificial solution is being examined. At pH 6, pyrophosphate (10^{-4}) inhibits while silicates (SiO_3) facilitate precipitation of brushite.

Studies in progress include:

(a) Physicochemical characterization of bone mineral from pathologic states. Bone biopsies have been obtained from patients suffering from osteoporosis, osteomalacia, hyperparathyroidism and Paget's disease of bone. These will be extracted with ethylenediamine to obtain bone mineral. Ionic exchange with ^{45}Ca and $^{32}PO_4$ will then be carried out.

(b) Calcification of reconstituted bone collagen. Soluble collagen from bone of patients with various disorders will be extracted and reconstituted. Factors controlling the calcification will then be analyzed.

Publications:

None.

Serial No. NHLI-239(c)
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Marvland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. II. New concepts in the pathogenesis and treatment of renal stones of calcium phosphate origin.

Previous Serial Number: NHLI-112(c)

Principal Investigator: Pak, C.Y.C., M.D.

Other Investigators: Ruskin, B. and Runkle, B.

Cooperating Units: Eanes, D., Ph.D., Laboratory of Biological Structure, NIDR.

Project Description:

Objectives:

The purpose of this study is two-fold: to examine the physicochemical factors concerned in the pathogenesis of renal stones containing calcium and to evaluate the efficacy of various forms of treatment.

Methods Employed:

The degree of saturation of urine with respect to brushite was calculated as follows. The activity product (K_{sp}) of Ca (2+) and HPO_4(2-) was calculated before and after incubation of urine with brushite. The ratio of initial and final activity products ($K_{sp,i}/K_{sp,f}$) represented the degree of saturation of urine where a value greater than 1 indicates supersaturation and value less than 1 undersaturation. The activity product ratio of calcium oxalate was calculated similarly.

The formation product of brushite from spontaneous precipitation was calculated as follows. The concentration of calcium in urine was raised by the addition of $CaCl_2$ while pH was kept constant. The point of spontaneous precipitation was determined after two days of incubation at 37°. It is expressed as the formation product ratio, or the number of times the specimen is supersaturated with respect to brushite when precipitation commences.

Major Findings:

Renal stones probably form by spontaneous precipitation (crystallization theory) or under the influence of an organic matrix (matrix theory). For

either theory, a state of supersaturation with respect to the crystal nidus is essential. Our studies clearly show that brushite ($CaHPO_4 \cdot {}^2H_2O$) constitutes the nidus of stones of calcium phosphate origin. First, brushite was identified from the precipitate formed from 12 urine specimens from normal subjects and 12 from patients with nephrolithiasis by the addition of calcium chloride. Second, some of the specimens which were supersaturated with respect to brushite calcified the modified collagen from Achilles tendon. The solid phase formed was brushite.

The formation product for the spontaneous precipitation of brushite from urine was shown to be two to 3.5 times the solubility product. Thus, the urine was 2 - 3.5 times supersaturated before brushite formed spontaneously. The formation product of "normal" urines was not significantly different from that of "stone-forming" urines. It is therefore concluded that the inhibitors of calcification do not play a significant role in the pathogenesis of renal stones, even though they probably increase the formation product.

Brushite serves as the nidus for stones of "hydroxyapatite" and calcium oxalate. At pH greater than 6.9, brushite undergoes hydrolysis to hydroxyapatite. At all urinary pH, brushite induces crystallization of calcium oxalate, not vice versa.

An essential requirement for the formation of calcium-containing renal stone is a state of supersaturation of urine with respect to brushite. It was shown that patients with hypercalciuria and stones usually excrete urine specimens which are supersaturated, whereas normocalciuric subjects usually do not (ref. 2 and 3). Patients with hypercalciuria and stones pass urine specimens which are supersaturated with respect to calcium oxalate as well; normocalciuric subjects generally do not. The important determinant of renal stone formation is the excessive urinary excretion of calcium.

The aim of therapy was therefore aimed at lowering the degree of saturation of urine with respect to brushite. Alternatively, it should be directed at raising the formation product, that is, widening the metastable region. Cellulose phosphate (1) has been shown to be the most effective agent, according to above criteria. It markedly decreased the urinary excretion of calcium and thereby lowered the activity product of brushite and of calcium oxalate. Sodium phosphate increased the formation product of brushite; however, it also increased the activity product of brushite, without significantly altering the activity product of calcium oxalate.

Fifteen patients are being followed on cellulose phosphate therapy for up to 2.5 years. Two patients have shown decrease in size or dissolution of stones. Every patient has experienced a dramatic subjective improvement. No significant side-effect has been uncovered.

Publications:

1. Pak, C.Y.C. and Ruskin, B.: Calcification of collagen by urine in vitro: Dependence on the degree of saturation of urine with respect to brushite. J. Clin. Invest. 49: 2353-2361, 1970.

2. Pak, C.Y.C., Cox, J.W., Powell, E. and Bartter, F.C.: Effect of the oral administration of ammonium chloride, sodium phosphate, cellulose phosphate and parathyroid extract on the activity product of brushite in urine. Am. J. Med. 50: 67-76, 1971.

3. Pak, C.Y.C., Eanes, E.D., and Ruskin, B.: Spontaneous precipitation of brushite in urine: Evidence that brushite is the nidus of renal stones originating as calcium phosphate. Proc. Nat. Acad. Sci. Accepted for publication.

Serial No. NHLI-240(c)
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism III. Mode of action of thyrocalcitonin.

Previous Serial Number: NHLI-115

Principal Investigators: Pak, C.Y.C., M.D. and Middler, S., M.D.

Other Investigators: Casper, A.G.T. and staff; Delea, C. and staff

Project Description:

Objectives:

The purpose of this study is to determine the effect of thyrocalcitonin (TCT) on: a) the renal transport of calcium and phosphorus in the dog, and b) on the gastrointestinal absorption of calcium in man.

(a) Methods Employed:

Four one-hour renal clearance periods with and without calcium infusion were performed in female mongrel dogs for inulin, calcium and phosphorus. From these, percent tubular resorption of calcium (TRCA) and phosphorus (TRP) were calculated. Intact and thyroparathyroidectomized dogs were studied.

After control and calcium infusion clearances, each dog underwent treatment with TCT (8 MRC u/kg/day) for up to 10 weeks. Renal clearance studies, again with and without calcium infusion were repeated at varying intervals during treatment.

Major Findings:

In intact dogs, TCT administration resulted in a reduction of TRP and an increase of phosphorus clearance. In some of them, these changes were accompanied by hypercalciuria. Response to calcium infusion remained normal even after 10 weeks of treatment. Serum alkaline phosphatase and urinary hydroxyproline did not change.

In contrast, TCT administration did not affect renal transport of phosphorus in thyroparathyroidectomized dogs. As in the intact group, the response to calcium infusion was not altered.

These results suggest that the effect of TCT on TRP and phosphorus clearance is not primary, but rather is mediated through stimulation of PTH. This implies then that chronic stimulation of PTH by TCT administration may produce parathyroid hyperplasia and/or adenoma. The normal response to calcium infusion in the intact dogs undergoing treatment is compatible with recent reports that PTH production by hyperplastic glands is suppressable.

Results of the histologic examination of the parathyroids and the microradiographic studies of bone are pending.

(b) Methods Employed:

Gastrointestinal absorption of calcium was determined by the procedure dependent on the external counting of the forearm 4 hours after an intravenous, and on a separate test after an oral dose of ^{47}Ca. Patients with post-surgical hypoparathyroidism were evaluated before and after administration of thyrocalcitonin (3-8 MRC u/Kg/day) for 3 to 4 days.

Major Findings:

The gastrointestinal absorption of calcium increased by 25 to 30 per cent after thyrocalcitonin treatment. Since the studies were performed in patients without parathyroid glands, the results suggest that TCT at pharmacological dosages directly stimulates the gastrointestinal absorption of calcium.

Publications:

1. Pak, C.Y.C., Ruskin, B. and Casper, A.G.T.: Renal effects of porcine thyrocalcitonin in the dog. Endocrinology 87: 262-270, 1970.

2. Pak, C.Y.C. and Bartter, F.C.: Renal effects of Vitamin D. In DeLuca, H.F. and Suttie, J.W. (Ed.): The Fat-Soluble Vitamins. Madison, Wisc. University of Wisconsin Press, 1970, pp. 101-110.

Serial No. NHLI-241
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. IV. Interaction of hormones at the air-water interface.

Previous Serial Number: NHLI-116

Principal Investigators: Kafka, M.S., Ph.D., and Pak, C.Y.C., M.D.

Project Description:

Objectives:

The purpose of these studies is to examine molecular interactions between peptide hormones and lipid monolayers. The interaction between hormone and monolayer may serve as a model for hormone action at the outer aspect of the cell plasma membrane. Previously, the actions of insulin and parathyroid hormone on Ca^{2+} adsorption at monolayers were studied, followed by studies of the effects of insulin analogues, vasopressin, oxytocin, thyrocalcitonin, and 3'5' cyclic AMP on Ca^{2+} adsorption at the monooctadecyl phosphate (mOP, stearic monophosphate) monolayer. Then the effects of insulin and vasopressin on the passage of water through monolayers were studied and have now been completed.

Methods Employed:

Molecular interactions between hormone and monolayer were studied in a modified Langmuir trough. The lipid monolayer was spread on the surface of sodium phosphate buffer containing $CaCl_2$ and, where appropriate, $^{45}CaCl_2$ in trace quantities, and compressed to 19.7 dyne cm. Surface pressure was maintained constant throughout the studies, and after 15 minutes of film stabilization reactants (hormone, diluent as control, and Prostaglandin E were introduced beneath the film.

Radioactivity at the monolayer was measured by a Geiger-Mueller Gas Flow Tube fixed above the trough. Changes in the surface area occupied by the monolayer were measured on a scale running along the side of the trough. Water passing through the monolayer was absorbed by dessicant fixed above the surface of the trough and was weighed at intervals. Specific resistance to evaporation, a reciprocal of the evaporation rate was calculated.

Major Findings:

The hypothesis that insulin and vasopressin increase the permeability of cells to water and water-soluble substances by decreasing the adsorption of Ca^{2+} at the outer surface of the plasma membrane was tested. Changes in the rate of evaporation of water through the monolayer were compared with changes in Ca^{2+} adsorption.

1) Insulin and vasopressin cause increased passage of water through the monolayer.

2) The increased water passage could be accounted for in part by inhibition of Ca^{2+} adsorption at the monolayer.

3) Increasing passage of water through the monolayer was specific for insulin and vasopressin.

4) Oxytocin and albumin caused the rate of water passage present at the time of their introduction to remain fixed, neither rising nor falling subsequently. Their modes of action in vivo may be related to these effects and may depend on their prevention of any change in Ca^{2+} adsorption in their presence.

5) Insulin and vasopressin increase the passage of water through the monolayer to a greater degree than the increase seen in the total absence of Ca^{2+}. In addition, changes in the rate of water passage do not always parallel decreases in Ca^{2+} adsorption in the presence of these hormones. Insulin and vasopressin, then, have in addition to their action in inhibiting Ca^{2+} adsorption, direct interaction with monolayer molecules.

a) Interaction between insulin and vasopressin and the monolayer do not depend on the presence of phosphate groups nor negatively charged groups.

b) The interaction is not electrostatic.

6) Prostaglandin E_1 changes neither the rate of water passage through the monolayer nor the adsorption of Ca^{2+}.

Publications:

None.

Serial No. NHLI-242(c)
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. V. Clinical implications of 25-hydroxycholecalciferol (25-HCC).

Previous Serial Number: NHLI-117(c)

Principal Investigators: Pak, C.Y.C., M.D., Delea, C.S., A.B., and Bartter, F.C., M.D.

Cooperating Units: DeLuca, H., Ph.D., Department of Biochemistry, University of Wisconsin

Project Description:

Objectives:

The purpose of this project is to evaluate the effect of treatment with 25-HCC of patients with Vitamin D resistance.

25-HCC is believed to be the biologically-active metabolite of vitamin D_3. Indeed, patients with vitamin D resistance frequently exhibit defective synthesis of this compound.

Methods Employed:

Patients who have shown clinical resistance to treatment with vitamin D_2, vitamin D_3 or dihydrotachysterol were screened for 25-hydroxylation. These patients were then treated with 25-HCC. Effect of treatment was evaluated on metabolic regimen from changes in serum calcium and phosphorus concentration, urinary calcium and phosphorus excretion, and in the gastrointestinal absorption of calcium.

Major Findings:

Four patients with Vitamin D-resistant hypoparathyroidism have been evaluated. The first patient was resistant to large doses of vitamin D_2, vitamin D_3 and dihydrotachysterol. She responded to 25-HCC (5000 u/d or less) with a significant increase in serum calcium concentration, and in urinary excretion of calcium. These changes were accompanied by an increase in urinary hydroxyproline excretion and a moderate increase in the gastrointestinal absorption of calcium. Two other patients with hypoparathyroidism responded to 25-HCC, with an increase in serum concentration of calcium, urinary calcium excretion, and in the gastrointestinal calcium absorption.

The fourth patient with hypoparathyroidism had a moderate resistance to vitamin D. She responded to 25-HCC by an increase in urinary calcium excretion. However, there was no significant change in serum Ca concentration. The lack of response in this patient may be the result of her moderate renal failure.

Three patients with vitamin D-resistant rickets have been treated with 25-HCC. Two showed a significant increase in serum P concentration and in the gastrointestinal absorption of calcium, and positive calcium and phosphorus balances. However, the third patient developed resistance to 25-HCC during treatment.

Future studies:

The exploration of the molecular basis for vitamin D resistance is being planned. The assay for 25-hydroxylase activity (from liver biopsy specimen) and for calcium-dependent ATP'ase from gut mucosa is sought.

Publications:

1. Pak, C.Y.C., and DeLuca, H.: Treatment of hypoparathyroidism with 25-hydroxycholecalciferol. In DeLuca, H.F. and Suttie, J.W. (Ed.): The Fat-Soluble Vitamins. Madison, Wisconsin., University of Wisconsin Press, 1969, pp. 173-176.

2. Pak, C.Y.C., DeLuca, H.F., Chavez, J.M., Suda, T., Ruskin, B. and Delea, C.S.: Treatment of vitamin D-resistant hypoparathyroidism with 25-hydroxycholecalciferol. Archives of Internal Medicine 126: 239-247, 1970.

Serial No. NHLI-243(c)
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. VI. Treatment of osteoporosis with calcium infusion and thyrocalcitonin.

Previous Serial Number: NHLI-111(c)

Principal Investigators: Middler, S.A., M.D., Pak, C.Y.C., M.D. and Bartter, F.C., M.D.

Cooperating Units: Henneman, D., M.D., Ortho Research, Raritan, N.J., Jowsey, J., M.D., Mayo Clinic, Rochester, Minn., Arnaud, C., Mayo Clinic, Rochester, Minn., and Murad, F., M.D., University of Virginia, Charlottesville.

Project Description:

Objectives:

Part of this study was discussed in previous project report NHLI-111(c). Osteoporosis is a common, disabling disease of bone for which there is, at present, no generally accepted mode of therapy or theory as to etiology. We have theorized that some forms of osteoporosis may be due to relatively too much parathyroid hormone (PTH) and/or too little thyrocalcitonin (TCT). This study involves the evaluation of calcium infusions and thyrocalcitonin, singly and combined, as therapeutic modalities in the treatment of osteoporosis The data obtained during the course of this treatment should add a great deal of insight as to the precise etiologies of this disease.

Methods Employed:

Patients to be studied are admitted to the Clinical Center for a period of 2-3 months. Some are asked to return for several different studies. The patients undergo calcium and phosphorus balance studies, including an estimation of endogenous fecal calcium by ^{47}Ca tracer techniques, before, during and after treatment. Treatment consists of 12 days of calcium infusions (15 mg/Kg/4 hours), thyrocalcitonin (1-8 MRC unit/Kg I.M.) or both.

In addition to balance data, hydroxyproline and cyclic AMP excretion is followed. The former is an indicator of bone turnover, and the latter reflects, in part, a renal action of PTH. Bone biopsies are performed for histologic diagnosis and microradiography and bone cell metabolism. Post-treatment biopsies are obtained when feasible. Serum has been collected and stored for PTH assay by Dr. Arnaud.

Major Findings:

The first 12 patients to receive calcium infusion alone have been reported in detail. About one-half responded with an improvement in calcium balance, which in some cases has lasted as long as 18 months. This was generally due to an improvement in fractional calcium absorption. Hydroxyproline excretion decreased during and after calcium infusions. Bone resorption area, as measured by microradiography, decreased in 10 patients examined, while bone formation area increased in 7.

The suppression of phosphorus excretion on the first day of calcium infusions was less than normal in 9 of these patients. This suggests some autonomy in parathyroid function. This finding is being reevaluated by measuring phosphorus excretion in fractionated 12-hour urines. We have found this to be a more sensitive indication of parathyroid suppressibility than 24-hour phosphorus excretion.

More recent studies have demonstrated that calcium infusion causes a fall in cyclic AMP excretion. In one patient it decreased from 2.8 to 2.0 umoles/day with a post-infusion "rebound" to 3.4. In another patient, repeated 4-day periods of calcium infusion consistently reduced cyclic AMP excretion from about 6.0 to about 3.0 umoles/day.

Studies presently in progress have revealed that thyrocalcitonin alone did not significantly affect calcium balance or induce negative balance, in two patients with osteoporosis. Also, thyrocalcitonin combined with calcium infusions had no advantage over calcium infusions alone. Whereas calcium infusion suppressed cyclic AMP excretion, TCT increased it in 2 of 3 patients, suggesting a secondary stimulation of parathyroid hormone secretion. When TCT and calcium infusions were given concurrently cyclic AMP excretion decreased.

Data are presently being collected on the effect of TCT on cyclic AMP excretion in hypoparathyroidism. If our theory of secondary PTH stimulation is correct, these subjects should show no effect of TCT on urinary cyclic AMP.

Those patients whose cAMP excretion was studied after calcium infusion or combined TCT and infusion were "non-responders". That is, they were among the 50% of osteoporotic patients who went into a more positive calcium balance with Ca infusions, but did not have a sustained post-treatment effect. Their cyclic AMP excretion also quickly rebounded to or above control levels. We are planning to study "responders" to see if the suppression of cAMP excretion is sustained in these individuals.

Publications:

1. Lifschitz, M.D., Pak, C.Y.C., Henneman, D., Jowsey, J., Pilch, Y. and Bartter, F.C.: Treatment of osteoporosis with calcium infusions. Trans. of Assoc. of Am. Phys. 83: 254-266, 1970.

2. Bartter, F.C.: Treatment of osteoporosis by calcium infusions. Proc. Royal Soc. Med. 63: 339-340, 1970.

Serial No. NHLI-244(c)

1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. VIII. Clinical applications of thyrocalcitonin (TCT).

Previous Serial Number: NHLI-119(c)

Principal Investigators: Middler, S., M.D., Pak, C.Y.C., M.D., and Bartter, F.C., M.D.

Other Investigators: Delea, C.S. and staff

Cooperating Units: Murad, F., M.D., University of Virginia, Charlottesville.

Project Description:

Objectives:

Thyrocalcitonin (TCT) is presently being used to treat Paget's disease of bone, hypercalcemia, and is being evaluated in the treatment of osteoporosis (see studies in Ca and P Metabolism. VI. Treatment of osteoporosis with Ca infusions and thyrocalcitonin). The mode of action of TCT per se must be separated from any secondary stimulation of parathyroid hormone which it may induce.

Methods Employed:

Patients with hypoparathyroidism are given TCT (2 MRC units/mg/d) for short courses and the gastrointestinal absorption of calcium is determined by means of an external counting technique. In addition, changes in urinary calcium, P and cyclic AMP excretion are followed.

Major Findings:

Two patients with hypoparathyroidism have been studied. TCT increased fractional calcium absorption from 57 to 63% and from 36 to 43%.

Future studies:

The above series will be extended, but the results suggest that TCT may augment calcium absorption from the gut independent of any parathyroid function. This raises the question of whether previous demonstration of augmented Ca absorption by parathyroid hormone was due to TCT stimulation. Patients with total thyroidectomies will be studied to see if parathyroid extract increases

their Ca absorption.

Publications:

1. Wills, M.R., Wortsman, J., Pak, C.Y.C., and Bartter, F.C.: The role of parathyroid hormone in the gastrointestinal absorption of calcium. Clin. Sci. 39: 89-94, 1970.

2. Wills, M.R., Zisman, E., Wortsman, J., Evens, R.G., Pak, C.Y.C., and Bartter, F.C.: The measurement of intestinal calcium absorption by external radioisotope counting: Application to study of nephrolithiasis. Clin. Sci. 39: 95-106, 1970.

Serial No. NHLI-245

1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. IX. Effect of diuretics.

Previous Serial Number: None.

Principal Investigators: Middler, S., M.D. and Pak, C.Y.C., M.D.

Other Investigators: Delea, C.S. and staff

Project Description:

Objectives:

Thiazide diuretics are widely used to treat hypercalciuric disorders associated with renal stones. This dose of drugs has been observed to decrease calcium excretion by 20-40%. The mechanism of action is obscure since most diuretics increase calcium excretion. This study is designed to help elucidate this mechanism, and to evaluate various diuretics in the treatment of hypercalciuric disorders of different etiologies.

Methods Employed:

Normal volunteers and patients with hypercalciuria (osteoporosis, hyper- and hypoparathyroidism, sarcoidosis, idiopathic hypercalciuria) are admitted to the Clinical Center for 2-4 weeks. They receive a constant metabolic diet of known composition. Urinary excretions of Ca, P, Na, K and cyclic AMP are followed. The last mentioned is believed to reflect the action of parathyroid hormone on the kidney. In addition blood is collected for assay of parathyroid hormone and thyrocalcitonin at a later date.

An estimation of the solubility product ratio with respect to brushite of the subject's urine is also made before and after treatment. Brushite is believed to be the nidus for the formation of calcium-containing renal stones.

Major Findings:

Control subjects decreased their urine calcium excretion by 24 to 38% on the fourth day of thiazide administration. Results in three patients with hypoparathyroidism on day 4 were -6%, -9% and +20% of the control. This suggests that thiazide diuretics affect urinary calcium excretion through a parathyroid-dependent mechanism.

Chlorothiazide, when given in a small dose to a hypercalcemic patient, caused a transient reduction in calcium excretion, but because of an increase in urinary pH, the activity product ratio actually increased. This may have been due to a carbonic anhydrase inhibition of the drug and prompted the study of other diuretics.

Furosemide in a dosage sufficient to cause a negative sodium balance equivalent to that produced by thiazides was found to have no effect, or actually to increase calcium excretion in two normal volunteers. This implies that negative salt balance per se is not the stimulus for renal calcium conservation.

Triamterene was given to two patients with primary hyperparathyroidism. In one it had essentially no effect on calcium excretion, although inducing a negative sodium balance of about 200 mEq. In the other patient, a fall in calcium excretion of about 30% occurred by the sixth day of treatment.

One patient with hypoparathyroidism was given Aldactone, 200 mg/day which increased urinary calcium excretion from 236 to 340 mg/d. Chlorothiazide was then given which decreased Ca excretion to 273 mg/d. This was probably due to a fall in filtration rate since creatinine clearance fell from 110 ml/min. to 64 ml/min. The serum calcium rapidly increased from 10.3 to 12.6 mg% after three days of chlorothiazide. This study suggests that chlorothiazide has a non-renal effect on calcium metabolism, which is not dependent on parathyroid function. Alternatively, the results may represent an increase in calcium absorption or bone resorption due to aldactone which could not be suppressed when renal calcium excretion was impaired by falling filtration rate.

Urinary cyclic AMP excretion has been measured in some patients receiving diuretics. In a patient with primary normocalcemic hyperparathyroidism and another with hypoparathyroidism there was no change in urinary cAMP with thiazide. Data are currently being collected on subjects with intact parathyroid function.

Publications:

None.

Serial No. NHLI-246(c)
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. X. Normocalcemic primary hyperparathyroidism.

Previous Serial Number: NHLI-120(c)

Principal Investigators: Pak, C.Y.C., M.D. and Bartter, F.C., M.D.

Other Investigators: Delea, C. and staff

Cooperating Units: Wells, S., M.D. and Ketcham, A., M.D., Surgery Branch, NCI; Tashjian, A., M.D., Boston, Mass.

Project Description:

Objectives:

The diagnosis of primary hyperparathyroidism in the vast majority of cases is based on the finding of an elevated serum calcium concentration with subsequent exclusion of all other causes of hypercalcemia. However, this entity, particularly when associated with renal stones, may present with serum calcium concentrations that are within the normal range. The purpose of this study is to examine the pathophysiology and the diagnostic criteria, and to assess the effect of parathyroidectomy.

Methods Employed:

1. Calcium infusion test. This consists of an intravenous infusion of calcium (15 mg/Kg) over a 4-hour period while on a constant diet. Urinary P and plasma PTH and TCT are monitored.

2. Plasma concentrations of PTH and TCT.

3. Urinary excretion of cyclic 3',5' adenosine monophosphate (cAMP).

4. Gastrointestinal absorption of calcium.

5. Urinary activity product of brushite.

Major Findings:

Twenty cases of normocalcemic primary hyperparathyroidism have been followed. Preoperatively, they show normocalcemia, normophosphatemia, normal serum alkaline phosphatase activity, and no obvious bone disease, except in one with osteoporosis. In addition, they show 1) hypercalciuria (urinary calcium of greater than 200 mg/d on a 400 mg Ca diet), 2) elevated plasma PTH in four in whom it was measured, 3) elevated plasma TCT in some (4/6), 4) enhanced gastrointestinal absorption of calcium, 5) supersaturation of urine with respect to brushite, and 6) an abnormal response to an intravenous infusion of calcium compatible with an "autonomous" hypersecretion of PTH.

Following removal of parathyroid adenoma or hyperplasia, the above abnormal findings return towards normal. Subjectively, the patients cease to form renal stones.

Publications:

1. Sanzenbacher, L.J., East, D.A., Pak, C.Y.C., and Bartter, F.C.: Preoperative and postoperative evaluation of patients with normocalcemic primary hyperparathyroidism. Surgical Forum 21: 1970.

Serial No. NHLI-247(c)
1. Endocrinology Branch
2. Section on Mineral Metabolism
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies in calcium and phosphorus metabolism. XI. Calcium infusion: diagnostic value, physiological consequences and effect on calcium homeostasis.

Previous Serial Number: NHLI-123(c)

Principal Investigators: Pak, C.Y.C., M.D., Sanzenbacher, L., M.D. and Bartter, F.C., M.D.

Other Investigators: Delea, C. and staff

Cooperating Units: Murad, F., M.D., U. of Va., Charlottesville, Va., and Arnaud, C., Ph.D., Mayo Clinic, Rochester, Minn.

Project Description:

Objectives:

The secretion of PTH and TCT is normally regulated by the serum concentration of circulating calcium ion. An increase in serum calcium concentration inhibits the secretion of PTH and stimulates that of TCT. A reduction in serum calcium concentration stimulates the secretion of PTH and inhibits that of TCT. The purpose of this study is to examine in detail the various consequences of induced hypercalcemia resulting from calcium infusion, e.g., effects on mineral metabolism and changes in calcium homeostasis dependent on PTH and TCT.

Methods Employed:

(A) Calcium infusion test was performed as follows. The patient was placed on a constant diet for four days. On day 3, he received calcium (15 mg/kg) intravenously over four hours from 9 AM to 1 PM. Urine was collected in 12-hour samples from 9 AM to 9 PM, and from 9 PM to 9 AM. Urinary Ca, P, hydroxyproline and cyclic AMP were determined.

Plasma PTH (immunoreactive) was measured before and after 4 hours of calcium infusion on the infusion day. More frequent measurements were made in selected patients.

(B) Effect of calcium infusion on the secretion of PTH and TCT was measured by the determination of urinary cyclic AMP and hydroxyprolines and by serum concentrations of TCT and PTH (by immunoassay).

Major Findings:

(A) Diagnostic value of calcium infusion. One of the frequently used tests for the diagnosis of hyperparathyroidism is the calcium infusion test. In hyperparathyroidism, the secretion of PTH is autonomous of normal control by changes in serum calcium concentration. Thus PTH secretion is not inhibited by calcium infusion. In the classical calcium infusion test, urinary P is used as a measure of parathyroid function. The failure of calcium infusion to suppress urinary excretion of P is regarded as an abnormal response.

Unfortunately, the classical calcium infusion test is subject to errors due to the non-specific effect of calcium infusions. An increase in the serum concentration of calcium resulting from calcium infusion increases the renal clearance of P, independently of PTH or TCT.

A modified calcium infusion test was devised which overcomes the non-specific effect of calcium infusion. It was noted that the period of hypercalcemia after a 4-hr. calcium infusion is usually less than 12 hours. The urinary P excretion during the second half of the infusion day is compared with that of the second half of the control day. Among normal subjects, the decrease in urinary P excretion was 51.5 $\pm$ 16.7 (S.D.)%. For 20 patients with hyperparathyroidism, the change in urinary P was -7.7 $\pm$ 14.1 (S.D.)%; all the values were above the mean + S.D. of the normal subjects. After parathyroidectomy, the change in urinary P was similar to that of normal subjects (-57.6 $\pm$ 18.8% (S.D.)).

The validity of the modified calcium infusion test was verified by serum PTH. Thus, in control subjects, PTH secretion was suppressed for more than 24 hours following calcium infusion. In contrast, patients with hyperparathyroidism show only a transient fall or no decrease in PTH secretion.

(B) Effect of calcium infusion on PTH activity. There is considerable evidence that calcium infusion normally causes a prolonged suppression of PTH secretion. First, after a single 4-hr. calcium infusion, urinary excretion of P and P clearance is suppressed for 12 hours after the serum calcium concentration returns to the preinfusion values. This conclusion was confirmed by direct radioimmunoassay of PTH. Secondly, after multiple calcium infusions (as in the treatment of patients with osteoporosis), there is a sustained decrease in serum Ca and P, and in the urinary excretion of total hydroxyproline and cAMP. This is probably the result of endogenous PTH and TCT since it is not observed in patients without thyroid and parathyroid glands. It is postulated that calcium homeostatic mechanism, dependent on PTH and TCT, may be altered by prolonged calcium infusions. This may have therapeutic significance in those conditions which result from an imbalance in the secretion of PTH and TCT, such as idiopathic osteoporosis.

Publications:

None.

Tech Development

Molecular Disease

Chemistry

ANNUAL REPORT OF THE
LABORATORY OF CHEMISTRY
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

The laboratory of Chemistry continues to pursue instrumental approaches to organic structural analysis. The group is increasingly convinced that classical chemical degradative approaches to structure are today seldom justifiable. For this reason, the laboratory has acquired the latest in high resolution nuclear magnetic resonance equipment (Varian XL-100) and is in the process of setting up an X-ray diffraction section under Dr. J. V. Silverton. In collaboration with him three members of the laboratory are completing X-ray structural analyses of (1) an oxidative dimer of trimethylphloroglucinol, (2) a mold pigment, and (3) an alkaloid of unusual structure. The X-ray facility will also have provision for low-angle scattering experiments on protein solutions.

Thus our present approach to structural analysis of a natural product, drug metabolite, or biochemical intermediate is as follows:

1. Procurement of the mass spectrum to determine formula and molecular weight (if applicable, gas chromatography-mass spectrometry is used to assure purity). In many cases the molecular weight peak is vanishingly small and the spectrum may be exceedingly complex. When this occurs, chemical ionization mass spectroscopy, extensively developed in this laboratory, usually provides the molecular weight as well as considerable structural information.

2. When enough material is available, high resolution nmr may provide, with ms data, a complete structural analysis unless a totally new type of carbon skeleton is at hand. Sensitivity is not high ($\sim$ 100 µg) but Fourier transform methods considerably reduce the amount required. Use of ^{13}C nmr, with ^{13}C of natural abundance sheds light directly on the carbon skeleton itself and we intend to conduct considerable research in this area because of its obvious potential.

3. Finally, when the sample does not yield to the above rapid techniques an effort is made to purify and crystallize the product for a complete X-ray structural analysis.

In the past year members of the laboratory, either alone or in collaboration with others, have:

1. Studied the application of the europium shift reagent to a series of keto steroids. Considerable simplification of spectra is observed but each case must be studied in detail with a range of concentration of reagent.

2. Studied the reaction of imidazole and related compounds with formaldehyde to obtain information on the action of chymotrypsin (Dr. M. Marini and Dr. A. Dunlop, Northwestern University).

3. Evolved a decoupling method for analysis of the substitution pattern of methoxyls by nmr (Dr. U. Weiss and K. Weisgraber, NIAMD).

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4. Studied binding phenomena (with phosphorus nmr) of lysolecithin and lecithin with their apoproteins.

5. Delineated the constituents of Schinus terebinthifolius Raddi (Brazilian Pepper tree). This tree, widespread in Florida, is responsible for considerable dermatitis and respiratory irritation of inhabitants. Important constituents are masticodienonic acid and the corresponding hydroxy acid.

6. Isolated a new alkaloid from Capsicum frutescens L. Work on its structure is in progress.

7. Work on "euphorbiasteroid" has been discontinued since its structure has been revealed by other work to be 6,20-epoxy-lathyrol.

8. The structure of a new triol diterpene from Helichrysum dendroideum has been elucidated as a kaurene derivative.

9. The structure of the trihexoside accumulated by victims of Fabry's disease has been revised. It contains an α-galactoside linkage rather than the ß-linkage previously reported by Sweeley et al (Dr. H. Sloan and Dr. D. Fredrickson, NHLI).

10. Nmr investigations of argininosuccinic acid (deposited by victims of argininosuccinic aciduria) and 4-fluoroglutamic acid have been undertaken to aid in determining their conformations as they interact with enzymes. The former has displayed a remarkably rigid form in aqueous solution.

11. The X-ray structure of $\Delta^{8,14}$-7α, 15ß, 22α, 23α-tetrachloroergosten-3ßyl acetate has been determined by an automatic phase solution method developed by Dr. J. V. Silverton and represents the first application of the method to an accentric crystal.

12. Considerable effort has been expended in the design of the new X-ray facility. A Nonius computer-controlled X-ray diffractometer, Wild-Heerbrug petrographic and steroscopic microscopes, Buerger precession and Weissenberg cameras, Dietzgen desk calculator, Siemens low angle scattering diffractometer and Nonius cryogenic cooling apparatus have been ordered.

13. The structure of gymnamine, a new lycodine alkaloid has been elucidated (G. Kapadia, Howard University).

14. Gigantine, an unusual peyote alkaloid, has been synthesized (G. Kapadia, Howard U.).

15. A series of new mescaline-derived α-amino acids has been discovered in peyote (G. Kapadia, Howard U.).

16. A series of aliphatic diamines has been synthesized to test the quasi-equilibrium theory of mass spectrometry in doubly-charged ions and to correlate charge separation with evolved energy. Evidence for discrete ionic electronic states has been obtained.

17. A software package has been written to allow the PDP-8/1 computer to acquire spectra directly from the LKB g.c.-mass spectrometer and to search a file for unknowns.

18. In collaboration with N. Law of Suburban Hospital, a procedure has been developed which utilizes GC-mass spectrometry to aid in the rapid identification (< 1 hr) of drugs ingested in overdose quantities. Members of the Laboratory and Mr. Law are available to four local hospitals around the clock for such analysis. In the past 9 months (July 70-April 71) 73 cases were investigated and 25 different drugs identified. Eighteen patients had ingested more than one drug and sometimes as many as six. Two patients died, one as the result of a fulminating infection, one as a result of a massive overdose of placidyl. The information is considered very useful by emergency room personnel and efforts are now being made to simplify the technique and to shorten analysis time.

In this connection, chemical ionization methods appear to be very promising. Most drugs give one peak corresponding to their molecular weight + 1, and as a result the mass spectrum of a mixture of drugs is enormously simplified. Because of this, the time-consuming gas chromatograph step can be eliminated. In 6 recent cases the results were found to be fully satisfactory.

19. The application of chemical ionization mass spectrometry to peptides has been investigated with promising results. Excellent spectra were obtained on the thyroid releasing factor, a tetrapeptide.

20. Chemical ionization spectra of barbiturates have been studied. The method provides essentially only quasimolecular ions for all barbiturates in contrast to ordinary electron impact spectrometry where molecular ions are not observed.

21. A series of plasticizers (diesters) has been studied with chemical ionization spectrometry. Using this method, their molecular weight may be easily determined and even differences in stereochemistry (maleic-fumaric, etc.) are revealed.

22. Coupling constants and chemical shifts using ^{15}N nmr have been studied in detail and correlations with structure derived (T. Axenrod, City College, N. Y.).

23. Phenylthiohydantoins resulting from the Edman Sequenator have been analyzed using deuterated internal standards and chemical ionization spectrometry. A new system has been proposed and will be acquired allowing semi-automatic analysis by repetitive scanning of a CI-quadrupole system (J. Pisano, B. Brewer, NHLI, and V. Aandahl, M. Shapiro, K. L. Ting, DCRT).

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Serial No. NHLI-248

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mass Spectrometry on Doubly-charged Ions

Project Serial Number: None

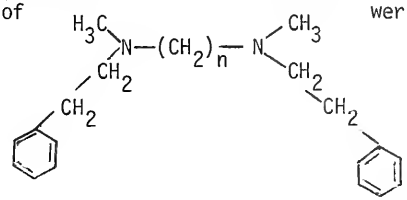
Principal Investigator: Kai-Li Ting, Ph.D., H. M. Fales, Ph. D.

Other Investigators: None

Cooperating Units: None

Project Description:

When a doubly-charged ion splits into two singly-charged ions in the mass spectrometer, it will give broad dish-shaped metastable peaks. The peak width indicates the kinetic energy released during the process which should correlate with the coulumbic repulsive energy between the two charges, so the intercharge distance can be calculated. A homologous series of compounds of the type of



were synthesized, using two nitrogens serving as the sites of charges. The intercharge distance can be measured from a chemical model. The kinetic energy released can be measured from metastable peak width by defocussing. The experimental result indicates that more complex processes occur simultaneously than simple charge expulsion. These energy data will give information of several fundamental processes in mass spectrometry.

Honors and Awards: None

Publications: None

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Serial No. NHLI-249
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland, 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Applications of nuclear magnetic resonance to biochemical or model bio-organic systems.

Previous Serial Number: None

Principal Investigator: E. A. Sokolcski, B. S.

Other Investigators: None

Cooperating Units: None

Project description:

During the past year four main projects were begun, each one dealing primarily with applications of nuclear magnetic resonance to biochemical or model bio-organic systems.

1. In collaboration with Drs. Weiss and Weisgraber (NIAMD) a study of methyl esters of aromatic systems was carried out. The investigators had observed some peculiar intensities of the methyl group in complicated pigment molecules isolated from natural sources and it was hoped that an explanation of the intensity discrepancies would lead to a method of assigning structures of these naturally occurring compounds. A number of substituted anisoles were examined in the nmr at various concentrations and temperatures. Decoupling experiments were performed which showed that approximately a 17% increase in intensity was observed when the ortho proton was irradiated, suggesting a coupling of this proton with the methyl group of the ether. A discrepancy still does exist since the original deviation from 100% was 25-28% of other methyl-ether protons in the pigment and at least one of the model systems. This 7-11% difference cannot be explained as of this time and further studies are necessary. A short communication was submitted to Texas A&M nmr News Letter. The method does seem to hold promise of use in situations where other techniques may fail.

2. This project was begun in collaboration with Dr. S. K. Devi (MDB/NHLI). Preliminary data on phosphorus nmr of lysolecithin and lecithin was obtained for studies of binding phenomena of apo-protein. With the recent acquisition of the XL-100-15 spectrometer, more significant data will be obtained and the work can continue in earnest and as a project of top priority.

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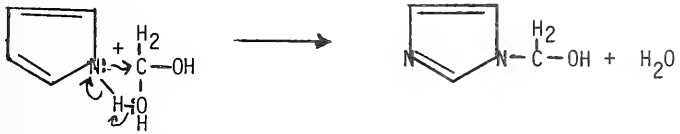
3. In this project the new shift reagent 2,2,6,6, tetramethyl-heptane-dionate-europium was used to simplify various sterols. Spectra of progesterone, 11 keto-progesterone, pregnenolone and ergosterol were obtained and shifts observed. Limited use of the method is seen, mainly where prior knowledge of expected spectral patterns is available or the unknown is of fairly simple structure with only one complexing site (OH, NH_2, C=O ...i.e., lone pair of electrons available). The shifts are sometimes more confusing than enlightening, and difficulties were encountered with sufficient solubility of shift reagent to give complete shift. The paramagnetic character of the Europium causes broadening of the spectra with loss of resolution. A new reagent recently available may solve the solubility problem making further work in the future more feasible.

4. The fourth project which is now awaiting preparation for submission for publication was done in collaboration with Dr. H. M. Fales of this laboratory and Drs. Morini and Dunlop of Northwestern University. To obtain some information of the action of chymotrypin, the reaction of imidazole with formaldehyde was chosen as a model system. Reactions of imidazole with aqueous formaldehyde were observed in situ in the nmr spectrometer at various pH's and temperatures. One of the possible mechanisms, that of orbital interaction of the imidazole, was shown to be most probably in error. The nmr showed that the site of interaction was the N-H of the imidazole.



The nmr spectra showed a new resonance peak at 5.6δ for the N-methylol system. Basic pH had little effect on the rate while acidic pH <2 caused a decrease in rate as would be expected from a protonated species:

Variable temperature studies showed that it was possible to observe the methylol species at low temperature pH∿7, the resulting spectral appearance closely resembling that of N-methyl imidazole.

In addition to these four projects, nmr data has been accumulated on several new natural products of Dr. Lloyd of this laboratory and future experiments are planned.

Publications: None

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Serial No. NHLI-250

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mass Spectrometric Methods of Analysis

Previous Serial Number: None

Principal Investigator: D. J. Pedder, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

A. Computerized Data Collection from the LKB-9000 GLC-MS Combination.

A software package has been written for the PDP8/I and LKB-9000. It acquires mass spectra, prints out the six largest peaks of each spectrum, and stores the spectra for later retrieval. The system is designed for maximum user convenience. It is particularly suited for the identification of drugs in overdose cases, as investigated here by Drs. Fales and Milne of this Laboratory together with Norman Law of Suburban Hospital and Virginia Aandahl of DCRT.

B. CI-MS Analysis of Biological Materials (with Dr. Milne of this Laboratory and Dr. Creveling of NIAMD).

Dansyl derivatives of amines are particularly amenable to TLC analysis for biochemical investigations. To minimize maniuplation and enhance sensitivity, direct mass spectral analysis is promising. Twelve chemical ionization spectra have been obtained on the modified MS-9. Preliminary results suggest that direct analysis of mixtures is feasible, as shown previously for the PTH derivatives of peptides.

Future Course : The software package will be extended by including a calibration routine, and adapting the current version of the poison file search program written by V. Aandahl (DCRT).

A less expensive, computerized CI-MS system is on order. It is expected that reprogramming it will be shared with Dr. Kai-Li Ting (DCRT). Once the

system is operational, the method will be used on PTH peptides, dansyl amines, permethylated peptides and other compounds of biological interest.

Honors and Awards: None

Publications: None

Serial No. NHLI-251

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: X-ray Crystallographic Structural Studies of Natural and Synthetic Compounds

Previous Serial Number: None

Principal Investigator: J. V. Silverton, Ph. D.

Other Investigators: None

Cooperating Units: None

Project Description:

Structural studies initiated before this reporting period have been continued and/or completed.

The structure of symmetrical cedrone (with H.M. Fales and J. Beisler, NIAMD) has been completed and the results are in press.

The structure of ruthenium red diamine hydrochloride has been completed (with P.M. Smith of Georgetown University) and the results are in press. This compound is related to the polysaccharide staining material ruthenium red and the structure should be helpful to an understanding of its action.

The structure of astrocasine (with H. A. Lloyd, NHLI and W. M. Bright, Georgetown University) is in the final stages of refinement and confirms Dr. Lloyd's chemical deduction of the structure.

The structure of $\Delta^{8,14}$-7α,15p,22α,23α tetrachloro ergonsten-3p-yl acetate (with P. M. Smith, Georgetown) is in the final stages of refinement. This structure, besides being of chemical significance because of its large O.R.D. effect, is also interesting in that it was solved by an essentially completely automatic phase solution method developed by the author and associates and represents the first solution by this method for an acentric crystal.

Data collection for cortisin (provided by Drs. K. Weisgraber and U. Weiss of NIAMD) is in progress and should be complete very soon. It is intended that this compound should be a first test of a new phase solution

program.

Considerable work has been carried out in developing the use of the symbolic addition method of Karle and Karle including the program referred to above: "Phase 2A". This has been tested on the known structure of symmetrical cedrone and has worked successfully. Work has also been carried out on the implementation of H. Hauptman's recent work on the phase solution for acentric crystals and is currently being tested on the very difficult problem of camphor quinone.

Much time has been spend on planning an X-ray crystallographic laboratory and the selection of instrumentation. It is hoped to have a versatile as possible data collection methods with reference especially to studying crystals only available at low temperatures as well as more normal crystals.

Honors and Awards: None

Publications:

Tsay, Y. H., Silverton, J. V., Beisler, J. A., and Sato, Y.: The structure of carpesterol. J. Amer. Chem. Soc., 92: 7005 (1970).

Langs, D. A., Silverton, J. V., and Bright, W. M.: The crystal structure of dimethyl sulfone. Chem. Commun., 1653 (1970).

Smith, P. M., Silverton, J. V., and Early, J. E.: The structure of ruthenium red. Inorg. Chem., In press (1971).

Beisler, J. A., and Silverton, J. V.: The crystal and molecular structure of symmetrical cedrone. Acta Cryst., In press (1971).

Beisler, J. A., Silverton, J. V., Penttila, A., Horn, D. H. S., and Fales, H.M.: The structure of the "Symmetrical Cedrone. J. Amer. Chem. Soc., 93, In press (1971).

Serial No. NHLI-252

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Application of Mass Spectrometry to Problems in Biochemistry

Previous Serial Number: NHI-233

Principal Investigator: G. W. A. Milne, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Work has continued on the technique of chemical ionization mass spectrometry. The design and performance characteristics of the MS-9 high pressure source have been established and the acquisition of a second chemical ionization system is in progress. Some exploratory work has been done in the area of negative ion chemical ionization mass spectrometry. It appears that the familiar reactions of carbanion chemistry can be studied in this way and more work in this area is planned.

The chemical ionization mass spectra of peptides have been found to give a great deal of sequence information. A computer program that automatically deduces a sequence from the mass spectrum has been developed and is in use. Peptides containing up to eight residues can be handled in this manner and if the amide nitrogens are methylated, larger peptides containing fifteen residues can often be sequenced very easily by this method.

Chemical ionization mass spectrometry is a very useful technique for the identification of dansyl derivatives of amines. The method is being used to identify the components of mixtures of amines derived from various tissues.

Honors and Awards: None

Publications:

Kiryushkin, A. A., Fales, H. M., Axenrod, T., Gilbert, E. J., and Milne, G. W. A.: Chemical Ionization of complex molecules, VI. Peptides. Organic Mass Spectrometry, 5, 19 (1971).

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Serial No. NHLI-253

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Chemical Investigation of Biochemical Reactions.

Previous Serial Number: NHI-232

Principal Investigator: G. W. A. Milne, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

The nmr spectra of compounds labeled with the stable isotope ^{15}N continue to receive attention. In anilines, the ^{15}N-H coupling and the ^{15}N chemical shift are related to the Hammett constant and in addition a three bond coupling between ^{15}N and the ortho protons has been discovered.

The chemical ionization mass spectra of a series of plasticizers have been measured and analyzed. Plasticizers, it has been found, can be identified with some confidence by this method and they have been found frequently in serum and in stomach contents of individuals. Attempts are being made to identify the origin of the plasticizers.

Honors and Awards: None

Publications:

Axenrod, T., Pregosin, P. S., Wieder, M. J., Becker, E. D., Bradley, R. B. and Milne, G. W. A.: Nitrogen-15 nuclear magnetic resonance spectroscopy. Substituent effects on ^{15}N-H coupling constants and nitrogen chemical shifts in aniline derivatives. J. Amer. Chem. Soc., 95, In press, 1971.

Serial No. NHLI-254

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Use of Digital Computing in Problems in Biochemistry

Previous Serial Number: NHI-231

Principal Investigator: H. M. Fales, Ph.D., G. W. A. Milne, Ph.D., V. Aandahl, B. S. and Kai-li Ting, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

An adequate file of electron impact mass spectra of some eighty drugs has been completed. A program to search this file has been written and has been in use for several months. The method has been used on a routine basis for nearly a year and has been very successful. About ninety overdose cases have been handled, mainly from Suburban Hospital, but also from Holy Cross, George Washington University Medical Center and Georgetown University Hospital. Accurate identification of the drugs involved has been possible in every case.

In order to effect faster identification of the drugs and also to lower the cost of the instrument, the chemical ionization mass spectra of some fifty drugs have been measured. As expected, these spectra consist generally of only one ion and so a computer is no longer essential to search the file. With such simple spectra, prior gas chromatographic separation of the components of a mixture is not necessary and the gas chromatograph can be eliminated.

Once the various compounds are in the mass spectrometer the mass spectrum is usually scanned. If, instead of a continuous scan, the spectrometer is switched from one mass to another, the whole process can be speeded up and since the identity of the compound follows from the switch settings of the mass spectrometer, there is no data handling to be done. This technique is successful in the identification of drugs and also of PTH amino acids formed by Edman degradation of proteins. Much of this work has been done manually and plans are now underway to acquire a quadrupole mass spectrometer which will be developed along these lines.

Honors and Awards: None

Publications:

Aandahl, Virginia, Fales, Henry M., Law, Norman C., and Milne, G. W. A.: Identification of dangerous drugs by mass spectrometry. Clinical Chemica Acta, 32, 221 (1971).

Fales, H. M. Nagai, Yumiko, Milne, G. W. A., Brewer, H. Bryan, Jr., Bronzert, Thomas and Pisano, John: The use of chemical ionization mass spectrometry in the analysis of the amino acid phenylthiohydantoin derivatives formed during the Edman degradation of proteins. Anal. Biochemistry, In press, 1971.

Fales, H. M., Milne, G. W. A., and Axenrod, T.: Identification of barbiturates by chemical ionization mass spectrometry. Anal. Chem., 42, 1432 (1970).

Serial No. NHLI-255
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Characterization of Natural Products

Previous Serial Number: NHI-230

Principal Investigator: H. A. Lloyd, Ph.D.

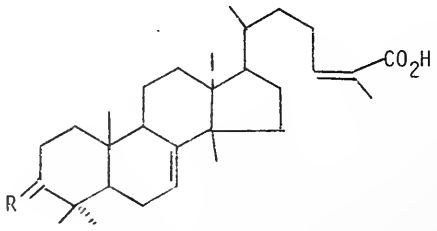
Other Investigators: None

Cooperating Units: None

Project Description:

Determinations of structure of Natural Products.

Schinus terebinithifolius Raddi. This tree, also called Brazilian pepper tree and Florida Holly, is widely spread through Florida. First introduced as an ornamental tree in the south of Florida, it has invaded the central and northern parts of the state and it has now become an unwelcome species. Dermatitis, respiratory and eye irritations have been blamed on its flowers and berries. The berries are also said to exert a paralyzing effect on birds upon ingestion. We are investigating the chemical constituents of the berries and attempting to determine what principles are responsible for these physiological actions. We have isolated a large number of sesquiterpenes and two pure crystalline triterpenes. These were characterized as the previously known compounds: masticodienonic acid (I) $C_{30}H_{48}O_3$ and the corresponding alcohol-acid (II)



I R = O
II R = H, OH

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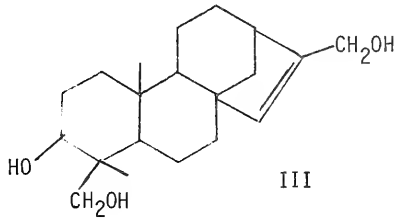
Other, more polar, materials have been isolated but they are still in an impure state. They appear to be triterpene acids or polyacids. We are also working on the isolation and structure of the coloring matter of the red berries.

Euphorbia lathyris. A crystalline sample (30 mg) of "euphorbiasteroid" was isolated and sent to us by Dr. Chakravarti (Indian Institute of Experimental Medicine, Calcutta). We have characterized the compound as a diterpene alcohol, 6,20-epoxylathyrol, which structure was determined recently through the collaboration of german, russian and french laboratories.

Capsicum frutescens L.(Bell pepper, green pepper). A phenolic alkaloid reported to exist in the plant in trace amounts has been isolated. Work on its structure is in progress.

Cicadas (17-year locusts). The discarded shells of the larvae of these insects have been extracted and yielded only a mixture of fatty acids to date.

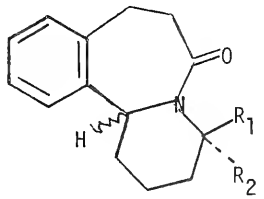
Helichrysum dendroideum. The work on the constituents of this plant was continued. The structure of a new triol diterpene was derived mostly from nmr evidence



III

Astrocasia phyllanthoides.

1) The conformations of the tricyclic degradation products (IV, a-d) of the main alkaloid astrocasine have been studied by nmr spectroscopy



IV

a $\begin{cases} R_1 = H \\ R_2 = CH_3 \end{cases}$

b $\begin{cases} R_1 = CH_3 \\ R_2 = H \end{cases}$

c $\begin{cases} R_1 = H \\ R_2 = CH_2OH \end{cases}$

d $\begin{cases} R_1 = CH_2OH \\ R_2 = H \end{cases}$

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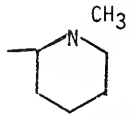
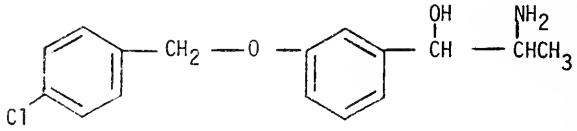
2) Work on the X-ray crystal structure of astrocasine

IVe $R_1 = H$ $R_2 =$ 

with Dr. J. V. Silverton has been pursued. The present results confirm the structure originally assigned through chemical degradation and reactions.

Metabolites of the drug MK 266.



This study in collaboration with Dr. H. Keiser (ET/NHLI) was initiated recently and no definite results have been achieved to date.

Work will be continued on all of the above projects.

Honors and Awards: None

Publications: None

Serial No. NHLI-256

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Characterization of Natural Materials

Previous Serial Number: NHI-229

Principal Investigator: R. J. Highet, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

1. In collaboration with Dr. Howard Sloan (MDB/NHLI), the glycoside linkages of glycolipids have been studied by nmr spectroscopy. By comparison with glycosides of known stereochemistry the nmr spectra of the intact glycolipids reveal the stereochemistry of these linkages; trihexoside accumulated by victims of Fabry's disease contains an α-galactoside and is not the all-β form previously reported.

A generally useful method of releasing the glycosides intact from the ceramide residue has been sought, so far without success.

2. In collaboration with Dr. Peter Goldman (A&R/NIAMD), an investigation of the nmr parameters of the isomers of 4-fluoroglutamic acid has been initiated. As these materials are known to serve as enzyme substrates, this study offers the possibility of determining the shape they assume when bound to an enzyme surface.

3. In further collaboration with Dr. Goldman, a study has been commenced of the chemistry of argininosuccinic acid, a material deposited by victims of argininosuccinic aciduria. Nmr spectra of the model substance, guanidinosuccinic acid, suggest that the material retains a remarkably rigid form in aqueous solution.

4. Early investigations of the utility of the XL-100 NMR spectrometer have revealed a design fault which must be corrected or circumvented to allow effective study of fluroaliphatic compounds. ^{13}C studies have been shown impracticable without decoupling equipment.

Future Course of Project:

1. Degradative methods providing intact glycosides from glycolipids in quantities suitable for nmr studies (ca. 1 mg.) will be sought. The investigations into the chemistry of argininosuccinic acid and the fluoroglutamates will be continued.

2. With the delivery of decoupling equipment for the XL-100 anticipated in the summer of this year, exploitation of ^{13}C nmr will be commenced by the study of the chemical shifts of phenol anions, methyl cyclohexane diols, and the various anhydrides of arginiosuccinic acid.

Honors and Awards: Appointed to Board of Editors of the Journal of Organic Chemistry.

Publications: None.

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Serial No. NHLI-257
1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Structure Elucidation, Synthesis and Biosynthesis of Natural Products

Previous Serial Number: NHI-228

Principal Investigator: G. S. Rao, Ph.D. with H. M. Fales, Ph.D.

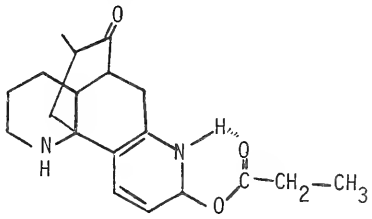
Other Investigators: None

Cooperating Units: None

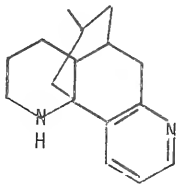
Project Description:

I. Structure elucidation of gymnamine, a new Lycodine alkaloid.

The Indian medicinal plant, Gymnema sylvestre R. Br. (Asclepiadaceae) is known to contain a hypoglycemic principle and the crude drug is being used in the treatment of diabetic patients in India. During a preliminary investigation of this active principle, Sinsheimer and McIlhenny (J. Pharm. Sci. 56, 732 (1967)) isolated a nonquaternary, nonphenolic alkaloid and partially characterized it. We have now studied the chemistry of this new alkaloid, designated gymnamine and have assigned structure I to the compound based upon its infrared, nmr, and mass spectra. The lycodine skeleton has



(I)



(II)

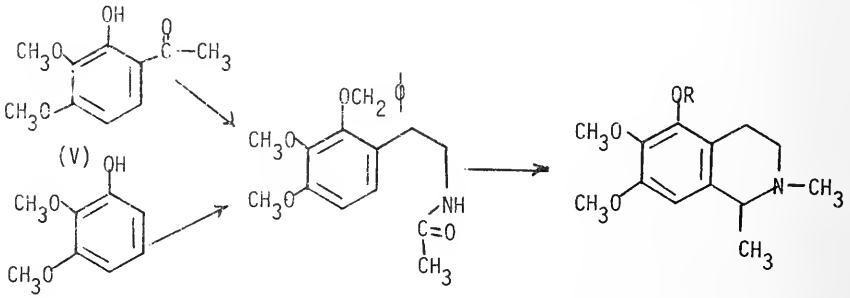
been confirmed by degradation of gymnamine to the known alkaloid, lycodine (II). The presence of uncommon propionic acid moiety (in ester linkage) has

also been confirmed by base hydrolysis and its identification as methyl propionate by GC-mass spectrometry.

This appears to be the first example of lycodine-type of alkaloid known to occur outside the Lycopodiaceae family. Gymnamine and related lycodine alkaloids are being tested for hypoglycemic activity at the University of Michigan.

II. Total synthesis of (±)-Gigantine (with Dr. G. J. Kapadia, Howard University).

In support of our recent revised structure for gigantine(III), a hallucinogenic alkaloid from the cactus Carnegia gigantea, we have now synthesized (±)-gigantine by a route different from that employed by us for the synthesis of (±)-O-methyl-gigantine (IV). The requisite intermediate VII was obtained



(VI) (VII)

(III) R = H

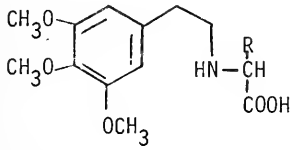
(IV) R = CH_3

beginning with either acetophenone V or phenol VI. The cyclization of VII furnished a Schiff's base which was quaternized with methyl iodide, reduced with sodium borohydride and hydrogenolyzed to afford (±)-gigantine in an overall yield of 34 percent. The synthetic base (III) was found to be identical with the natural alkaloid by tlc, glc, infrared, nmr, and mass spectrometry.

III. Identification of mescaloxylic acid and mescaloruvic acid, two novel amino acid analogs of mescaline, in the peyote cactus (with Dr. G. J. Kapadia, Howard University).

In a continuation of our studies on the peyote constituents, we have identified two new amino acid analogs of mescaline in the amino acid fraction of the cactus. The two novel compounds have been designated mescaloxylic (VIII) and mescaloruvic acids (IX) which are glycine and alanine analogs of

mescaline, probably formed via reductive deamination in the plant. The

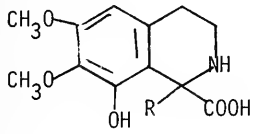


(VIII) R = H

(IX) R = CH_3

identification of the two acids was accomplished by gc-mass spectrometry of their TMS derivatives and by comparison to the synthetic reference compounds. The isolation of the new compounds was carried out by preparative paper chromatography.

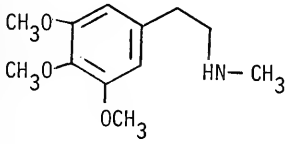
The two new amino acids identified in peyote are in contrast to the cyclic analogs (X) and (XI) recently reported by us. We have demonstrated



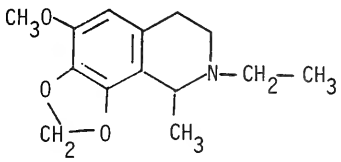
(X) R = H

(XI) R = CH_3

earlier the involvment of the cyclic compounds (X) and (XI) in the biogenesis of tetrahydroisoquinoline alkaloids of peyote. We are currently investigating the possible role of the open-chain compounds (VIII) and (IX) in the biosynthesis of peyote alkaloids, e. g., N-methyl-mescaline (XII) and peyophorine (XIII) which could result via reductive decarboxylation. The cycliza-



(XII)



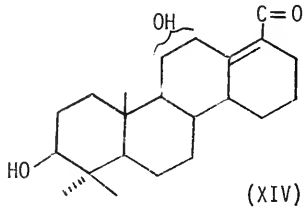
(XIII)

tion through pyruvate conjugation of the decarboxylated product of IX could lead to the unusual N-ethyl alkaloid (XIII).

Projects in Progress:

I. Structure elucidation of agaricusterol, a novel fungal metabolite.

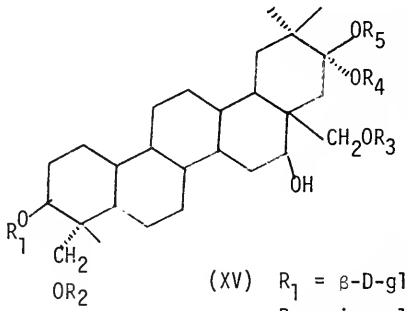
Additional chemical and spectral investigations of agaricusterol have confirmed the tentative structure (XIV) previously designated by us. Work is in progress to degrade chemically agaricusterol to a known reference compound.



(XIV)

II. Structure elucidation of gymnemic acid, the centisweet and antiviral principle of Gymnema sylvestre (with Prof. J. E. Sinsheimer, The University of Michigan).

In continuation of our investigation of gymnemic acid, we are now in the process of obtaining additional confirmatory proof for the proposed structure (XV) for the active principle.



(XV) R_1 = β-D-glucuronic acid
R_2 = isovaleryl
R_3 = tiglyl
R_4 = formyl
R_5 = acetyl

III. Biosynthesis of peyote alkaloids (with Dr. G.J. Kapadia, Howard University).

Investigations designed to elucidate the biosynthetic pathways leading to various peyote alkaloids are in progress. This involves synthesis of radioactive precursors, feeding to intact plants, and isolation and chemical degradation of the target products.

IV. Isolation and structure elucidation of constituents from the Brazilian Pepper (with Dr. H.A. Lloyd of this Laboratory).

We are currently investigating the constituents of Brazilian pepper with special emphasis on polar constituents and coloring pigments.

Honors and Awards: None

Publications:

Rao, G. S.: Identity of peyocactin, an antibiotic from peyote (Lophophora williamsi), and hordenine. J. Pharm. Pharmacol. 22, 544 (1970).

Kapadia, G. J., Fayez, M. B. E., Sethi, M. L., and Gao, G. S.: Synthesis of the tetrahydroisoquinoline alkaloids (±)-terpenine, tehaunine and (±)-o-methylgigantine and revised structure of gigantine. Chem. Commun., 856 (1970).

Kapadia, G. J., Rao, G. S., Leete, E., Fayez, M. B. E., Vaishnav, Y. N., and Fales, H. M.: On the origin of carbon 1 in tetrahydroisoquinoline alkaloids. J. Amer. Chem. Soc., 92, 6943 (1970).

Kapadia, G. J., Rao, G. S., Fayez, M. B. E., Chowdhury, B. K., and Sethi, M.L.: Total synthesis of (±)-gigantine. Chem. Ind. (London), 1593 (1970).

Serial No. NHLI-258

1. Laboratory of Chemistry
2.
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mass Spectrometry and Structure of Natural Products

Previous Serial Number: NHLI 227

Principal Investigator: H. M. Fales, Ph.D.

Other Investigators: None

Cooperating Units: None

Project Description:

Chemical ionization (CI) mass spectrometry continues to be studied in detail as it pertains to substances of biological origin. Thus, the simplification of spectra in the molecular weight region have allowed us to develop a new method for emergency drug identification and the analysis of PTH amino-acid derivatives in conjunction with the Beckman peptide sequenator. We have shown that peptides themselves are easily studied by CI methods, although the real difficulties usually surround the preparation of satisfactory derivatives. In the case of the thyroid releasing factor (TRF), the method worked well and provided excellent data relating to its structure (D. Desiderio, Baylor Univ.).

Artifacts (plasticizers, etc) are a continual source of trouble in extending mass spectrometry to lower levels in biological samples and we have recently applied mass spectrometry to their identification with very satisfactory results, particularly in the case of the phthalate esters.

Use of combined GC-MS has been fruitful in many areas at NIH and we have extended our previous studies on insect pheremones (M. Blum, Univ. of Georgia) to trail substances of the Manica beetle and Chrysomela larvae. Useful attractants (and repellents) may come from this work.

The metabolism of dapsone and ritalin are both being investigated (P. G. Dayton, Emory University) by GC-mass spectrometry and chemical ionization. Incorporation of radioactivity into urea has been observed in the former case.

Future Work:

A new chemical ionization-computer-quadropole mass spectrometer system, including a gas chromatograph, will be applied to the PTH analysis and other

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problems. Installation of a Fourier Transform nmr and ir system should assist us in development of a technique to allow infrared spectra to be run on GC eluates of biologically important compounds. Drug metabolism and insect pheremones studies will both be greated assisted by these capabilities.

Honors and Awards: None

Publications:

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Tech Development

Molecular Disease

ANNUAL REPORT OF THE
LABORATORY OF TECHNICAL DEVELOPMENT
NATIONAL HEART AND LUNG INSTITUTE
July 1, 1970 through June 30, 1971

The Laboratory of Technical Development is concerned with problems of basic and clinical medical science that can be solved by the development of new instruments and methods. Solution of these sorts of problems requires development of methods and evaluation of their utility by direct application to research or clinical situations. The laboratory provides an environment and facilities for instrumentation and method development in close conjunction with the medical research program of NIH. A section of the laboratory is specifically dedicated to research into the physiology of artificially supported pulmonary and cardiac function and to the development and application of support systems.

INSTRUMENTATION

Mechanized Microbiology

We have been investigating a new system for performing some of the common procedures in microbiology and cell counting. The system substitutes glass capillaries for the conventional petri dish. Microcolonies or cells are detected as light scattering particles when the capillary is passed through a light beam. We scan the capillary shortly after it is filled; this determines the baseline scattering pattern. Following incubation we rescan the tube. New or augmented scattering sources appearing in the later scans indicate growth. Size changes on the order of a cell diameter are easily detected so that initial results can be obtained in two or three hours, rather than a day or so. Antibiotics, essential growth factors or inhibitors can thus be evaluated in a matter of a few hours. We can handle a large number of samples with considerable saving of space and reagents using mechanized sample manipulation and electronic techniques for data processing.

Applications of our system include an antibiotic susceptibility assay, now undergoing clinical trial elsewhere, viable bacteria enumeration in urine samples, mycoplasma counting, and tumor cell studies. The response of hepatoma and myeloma cell lines to antimetabolites have been evaluated in less than two days. Relative antimetabolite effectiveness determined this way may be applicable to selection of clinical chemotherapeutic agents and rapid assay of new ones. Variations of the system are under development to facilitate automation, to reduce the number of cells needed to study and to accommodate immunochemical methods.

Instrumentation for Extracorporeal Support

Several instrumental methods have been developed in response to the needs of the Section on Pulmonary and Cardiac Assist Devices where long term perfusion of blood through the artificial lung and the patient requires continuous performance evaluation, rapid response and minimization of the blood consumed for analysis.

Oxygen saturation of the blood in the circuit provides good indices of the patient's condition and the performance of the oxygenator; the relatively high efficiency of the artificial lung permits rapid control of blood pumping and control of pCO_2. A mass spectrometer system has been evaluated and found satisfactory but price and complexity reduce clinical utility. We developed an oxygen saturation meter that monitors saturation through the wall of a blood conduit without sampling or even contacting the blood. The instrument, based on the principle of light reflectance, has been completed, tested and incorporated into the clinical pulmonary support system and has been shown to perform to requirements in animal experimental systems. Solid state light sources, sensors, and electronics contribute compactness, economy and dependability to the instrument. Additional instrumentation that exploits recent advances in electronics and materials are being developed for analysis of pH and electrolytes on microliter samples of soluble components diffused from the blood stream into a sample tube. This approach eliminates the problem of blood consumption by sampling and this advantage looms large in both research and clinical application.

Evaluation of cardiac function in animals has been made by constructing a special purpose analog computer with integrated circuits to compute stroke volume. Encouraging results to date make it likely that this instrument will become a valuable adjunct to experimental studies.

Blood Flow Measurement

Evaluation of regional blood flow in individuals that have incipent or even moderately advanced vascular disease is presently hampered by the requirement of invasive methods that usually do not permit serial measurements to determine progression or resolution of the disease. We have examined the feasibility of utilizing nuclear magnetic resonance to provide a means whereby the protons of blood can be induced to resonate in response to an electromagnetic field that freely permeates the tissues and to exploit the relatively long duration of the resonance condition (ca 0.4 sec. half life) to trace the path of the resonating protons. Experiments have established that the method has sufficient sensivity to trace flow in relatively small vessels even when the vessel contains only a small percentage of the total protons in the sensing field. A new system introduced last year will be studied as a means of measuring relative cerebral blood

flow by the Neurosurgical Laboratory at Marquette School of Medicine on contract to the Laboratory of Technical Development; we will continue to develop the instrumentation to improve sensitivity and specificity of the method. The proposed method polarizes protons of the blood in the heart and lungs by means of a high magnetic field. Then, while the sensitized protons flow through the brain they can be exposed in local regions to fields that will modify them so that the local marker will contribute a signal detectable in the effluent circulation. The method has been adequately tested in vitro but new approaches necessary to the in vivo demonstration are not complete at this time either in this laboratory or in the contract facility. The projection is that symmetrical regions in the brain can be compared in their ability to respond to CO_2 inhalation; thus we should have a non-invasive method for cerebral blood flow, a potential means to screen the population for those at maximum risk from cerebral vascular accidents.

CHROMATOGRAPHY

Chromatographic methods have contributed greatly to biochemical and pharmacological studies and it is generally evident that methods of even greater sensitivity, resolving power, speed and dependability are needed. Our current developments in chromatography are directed to the refinement of liquid-liquid chromatography by eliminating the solid support that is responsible for tailing and denaturation of sensitive materials. The most recent method consists of a continuous flow helix countercurrent chromatograph that permits continuous flow without rotating seals, while the helix is exposed to high centrifugal fields. We mount the helix in the centrifuge so the column counter-rotates at a rate equal to that of the revolution of the centrifuge; thin plastic inflow and outflow tubing can be connected directly to the column without twisting, and without rotating seals. A set of DNP labeled amino acids has been separated in analytical runs. A 10 µl sample of nine amino acids has been separated with an efficiency ranging from 3,000 to 10,000 theoretical plates. A preparative run handled 1 ml of the same material in 13 hours with 1,000 to 4,000 theoretical plate efficiency. The advantages of these methods include freedom from solid support effects and a small volume of distribution of each component that reduces detector sensitivity requirements. The method should also be applicable to macro-molecules, cells and other particulates.

FLUORESCENCE METHODS

Spectral shifts and polarization of fluorescence of DNS labeled lysozyme were used to determine the number of fluorescent dye molecules bound, the site of attachment and the degree of rotational freedom of the marker dye. The information can be correlated with X-ray crystallographic determinations of the three dimensional structure of the protein. Albumin-bilirubin

binding constants were determined by study of quenching of tryptophan fluorescence. The method illustrates how energy transfer as evidenced by quenching can be used to obtain accurate data about the number of molecules bound, the strength of binding and of photostability. Spectrofluorometric analysis of isomers of fluorescein isothiocyanate indicated that one had a greater quantym yield and a more favorable excitation wavelength. This provides definite evidence that one isomer is superior to the other for immunofluorescence work. Kinetics of staphylococcal nuclease renaturation were studied with stopped-flow fluorescence techniques. Changes in fluorescence due to concentration and solvent effects provided data on isoenzyme activity and lipoprotein structure. A study of the mechanism of mercury quenching indicated that this technique may be valuable for determining the number and location of sulfhydryl groups in proteins.

Stopped-flow methods for fluorescence, thermal, pH and absorption studies and calorimetric apparatus developed here have been applied to several biochemical problems in the Medical School at the University of California. The methods and instruments are being applied in active studies of enzyme kinetics and biochemical reactions. Modifications of the instrumentation are being made in response to additional requirements and as new technology becomes available.

PULMONARY AND CARDIAC ASSIST DEVICES

The spiral coil membrane oxygenator has been applied to extracorporeal blood oxygenation of alert unanesthetized animals for days to weeks without apparent morbidity attributable to the pump and oxygenator. The pulmonary support obtained by venous-venous perfusion can maintain gas exchange at near normal levels in absence of pulmonary function. Some patients with severe pulmonary disease have been supported temporarily with no apparent harm attributable to the support system.

Total support of the circulation was demonstrated by maintaining lambs in ventribular fibrillation on a veno-arterial pump oxygenator system for several hours. The animals were defibrillated and showed no morbidity attributable to the pump oxygenator system. It would appear that this latter system affords a method of relief of the damaged failing heart that is further compromised by failing pulmonary function due to pulmonary edema. It is considered that perfusion and oxygenation will offer greater potential for recovery than simple augmentation of the heart action when the pulmonary system is compromised.

The present developments include the improvement of membrane material by application of technology not currently used in membrane manufacture and the development of thin-walled non-kinking catheters using wire reinforcing and new materials. New blood

gas monitoring instrumentation has reduced the need for frequent sampling; gentle pumping and pump controls have minimized blood damage and the compact efficient system has reduced the need for priming blood. Blood loss by internal bleeding and leakage at catheter sites is being reduced by both meticulous surgery and decrease in heparin levels as perfusion proceeds.

Serial No. NHLI-259
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Indirect Cardiac Output Computation

Previous Serial No.: None

Principal Investigator: Gerald G. Vurek

Other Investigator: Roger G. Spragg

Cooperating Units: None

Project Description:

Objectives: Our aim is to assess the feasibility of using a small special purpose computer to calculate cardiac output from aortic pressure which should be useful in assessing the status of an acutely ill patient in an intensive care environment.

Methods Employed: Using the work of Fry and his colleagues at NHLI, we feel it is reasonable to assume that the load impedance of the heart consists of an elastic compliance of the aorta and a peripheral resistance. It can be shown that stroke volume inputs to this simplified impedance can be computed by summing the pressure and its derivative, appropriately scaled. We used the electrocardiogram signal to reset a special purpose analog computer at the end of each systolic period. The computer automatically changes its estimation of the peripheral resistance term at each resetting point so that the computed stroke volume is zero at the end of diastole. Therefore, the computer can accommodate changes in peripheral resistance and still provide information about the stroke volume of the heart.

We built a small special purpose analog computer using integrated circuits to perform the computation. Aortic pressures were measured with a Statham P23dE transducer connected to a 30 cm. #18 TFE catheter through a specially designed restrictor. The restrictor consists of a Kel-F screw with the threads partly removed; this "tunes" the catheter-transducer system for optimum frequency response, which in our case is flat to 35 Hz, with less than 6% overshoot response to a step pressure transient.

Sheep weighing between 10 and 15 kilograms were prepared with the Teflon catheter in the aorta via one carotid artery, a dyd sampling catheter in another carotid artery, and a dye injection catheter in the right atrium via a jugular vein. The

output of the computer and the aortic pressure waveforms were simultaneously recorded on a Sanborn recorder. Dye dilution curves were made at intervals and the cardiac outputs estimated by comparison with the computed cardiac outputs. We changed the animal's cardiac output by sterile exsanguination, reinfusion of the blood, breathing 10% oxygen/90% nitrogen, and with vasoactive drugs.

Major Findings: One set of experiments showed a correlation greater than 90% bewteen the computed output, the output measured by dye dilution over a 2 to 1 cardiac output range. These encouraging results have not been repeatable and we have not determined the difficulty. Part of the problem may be with the computer; part may be with the placement of the catheter. Since the computer must differientiate the signal it is susceptible to noise which may interfere with the resetting procedure. The catheter tip must be placed close to the aortic valve in order to obtain the proper pressure signal. This placement may not have been achieved in our procedures. Since it has been shown that the flow in the descending aorta can be computed with this approach we feel that the underlying assumptions are valid and we are continuing to try to determine the discrepancies between our computed results and the dyd dilution curves.

Significance to Biomedical Research and the Program of the Institute: One of the ways to assess the performance of the heart is by measuring the stroke-volume or cardiac output. Isolated measurements provide some information about the status of a compromised heart but continuous measurements or frequent measurement can provide trend information which should be useful in providing diagnostic information about hearts. Other workers have made monitoring systems based on average or otherwise processed aortic pressure waveforms assuming cardiac output is related to the average pressure and some calibrated peripheral resistance. These other schemes cannot accommodate changes in peripheral resistance and must be recalibrated with a dye-dilution system periodically. Our technique should be able to accommodate to changes in peripheral resistance. Central aortic pressure signals are usually available from patients in intensive care units, as is the ECG signal, so that the computation should require no significant additional burden on the patient. The computer can be housed in a box about 15 cm on a side, including transducer amplifier, so that space requirements are minimal.

Honors and Awards: None

Publications: None

Serial No. NHLI-260
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Title: Microdialyzer for Continuous Sampling of Small Molecules from Blood

Previous Serial Number: None

Principal Investigator: Gerald G. Vurek

Other Investigator: Theodor Kolobow

Cooperating Units: None

Project Description:

Objectives: The determination of the status and progress of severely ill patients requires biochemical measurements as well as physical measurements. Where prolonged extracorporeal support is part of the therapy, it is important to be able to make these measurements with as few samples as possible to reduce blood loss and the possibility of contamination. Our aim is to develop a dialyzer to remove small amounts of diffusable material from a portion of the blood in the extracorporeal circuit without breaking the sterile barrier and to develop suitable analytical techniques for the desired materials.

Methods Employed: Use use dialysis material manufactured by Dow Corning Corp. for their "capillary fiber" dialyzer. This material is tubular with 25 micron wall and 200 micron diameter. Blood passes over the outside of the tube and diffusable ions penetrate the wall, entering the aqueous "dialysate" compartment. The dialysate leaves the dialyzer and enters analytical instruments or a fraction collector for later analysis. We are currently using ion specific electrodes for the first stage of analysis: H+, Na+, k+ and Cl-. Dialysate flow rates from five to 50 µl/min. are used, althouth the typical rate is 10 µl/min.

Major Findings: At typical flow rates, equilibrium between blood and dialysate is at least 99% complete, within the error of measurement. Test solutions of buffer and albumin have been used to establish the effectiveness of the dialyzer and the electrode system. We tried to measure blood pH using a veno-arterial shunt installed in a sheep; results were inconclusive due to severe clotting problems, possible leaks in the membrane, and potential loss of CO_2 from the blood or dialysate before the

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solution reached the pH electrode. Work is in progress to establish the principal sources of difficulty.

Flow through electrodes have been made from commercial dip type electrodes by cementing silicone rubber tubing directly to the sensitive "membrane" of the electrodes. A small hole in the tubing permits the sample to contact the electrode. The sample size needed is less than 1 µl., except for the necessity to make contact with the reference electrode; this makes the practical volume about 5 µl.

Significance to Biomedical Research and the Program of the Institute: Changes in the chemical compositions of the body fluids reflect the status of an acutely ill patient in different ways than are indicated by physical measurements such as blood pressure, heart rate, ECG, Waveform, etc. The development of "non sampling" continuous monitors of body chemistry can provide a tool to make these measurements and establish their usefulness. In addition, they provide a potential for research studies on small animals since virtually no sample is taken from the animal.

Honors and Awards: None

Publications: None

Serial No. NHLI-261
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Title: Reflectance Oximeter for Continuous Measurement of Oxygen Saturation

Previous Serial Number: NHLI-186

Principal Investigator: Gerald G. Vurek

Other Investigators: Theodor Kolobow, W.S. Friauf

Cooperating Units: BEIB, DRS

Project Description:

Objectives: Our objective is to develop a non-contacting, non-sampling device for continuous monitoring of blood oxygen saturation. This measurement provides information about the metabolic status of animals of patients perfused with an oxygenator, as well as information about the oxygenator's performance.

Methods Employed: We use the difference between the optical properties of oxyhemoglobin and hemoglobin to indicate the blood saturation. Since part of our goal was to make a non-sampling, non-contact measurement we chose an optical system which would be reasonably independent of the surface of the tubing through which the blood flowed, as well as being able to accommodate large diameter tubing (1 cm). Our earlier instrument using reflectance techniques was susceptible to tubing surface quality so that we changed from reflectance to lateral "transmission" or diffuse scatter measurement. Light sources, detectors, and electronics are all solid state devices. We have used an American Optical Company oximeter to calibrate our instrument.

Major Findings: We have been able to exploit the optical "diffusion" properties of blood to make our oximeter measure saturation over the range of 50 to 98%, and be reasonably independent of hematocrit over the range of 20 to 55% red cell fraction. Light from the source enters the blood through the wall of the cuvette and diffuses in all directions; the receiver captures a portion of the light which escapes from the tube a few mm from the source. The signal depends on the geometry (inverse fourth power of distance), inversely with hematocrit, and inversely with saturation (at 650 nm). By measuring at two wavelengths and over two paths, we can cancel the geometry and

hematocrit effects and get a signal proportional to saturation. The earlier versions of our instrument used a logarithmic measurement to obtain saturation; more recent results indicate that if we compute the ratio of the signal in the infra-red (relatively independent of saturation) to the signal in the red, we can obtain a linear relation between saturation by our instrument and the A.O. standard over the range of 40% to 98%. We are currently designing the testing a simplified version which should be considerably easier and less costly to build; this would benefit users of the device because commercial development will be encouraged. Tests with earlier versions of the instrument have demonstrated its stability, reliability, and convenience.

Significance to Biomedical Research and the Program of the Institute: The flow-through reflectance oximeter provides a convenient means for continuously monitoring the oxygen consumption of acutely ill patients. The measurement is benign and no electrical apparatus comes directly incontact with blood. This instrument is one of a new series of continuous biochemical monitoring instruments for better assessment of the status of the acutely ill cardiovascular patient.

Honors and Awards: None

Publications: None

Serial No. NHLI-262
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Methodology in Fluorescence Measurements

Previous Serial No.: NHLI-183

Principal Investigator: Raymond F. Chen

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: Fluorescence spectroscopy is a powerful tool for studying structure of macromolecules, but this technique is still in its early adolescence. Thus, it is necessary to improve and refine various techniques of fluorescence assay as much as possible, taking into consideration that very different types of measurement are involved in determining decay times, quantum yields, spectra, and kinetics.

Methods Employed: As in the past, our measurements used Aminco-Bowman spectrofluorometers, the TRW lifetime apparatus, and stopped-flow devices supplied by the American Instrument Co.

Major Findings: The problems involved in fluorescence polarization determinations with a grating-type spectrofluorometer were reviewed. The grating factor is the ratio showing the relative efficiency of the detector system for vertically polarized light compared with horizontally polarized light. The factor was determined for one Aminco-Bowman instrument over a wide range of wavelengths. Interestingly, there were sharp changes in the factor, which ranged from above 1 to about 0.8. Another problem is how to correct for blanks when making polarization measurements. Blank correction is very important especially for solutions of low quantum yield. These findings will be reported and discussed in a paper now in preparation.

In relation to the controversy over quantum yields we have confirmed that the reported yields of quinine and fluorescein of 0.55 and 0.85 are in the right ratio. This proves to us that a recent paper advocating a value of 0.70 for quinine cannot be correct, since fluorescein would then have a yield greater than unity. Other efforts in this field include a better method for chromatography of rhodamine B.

A chapter on fluorescence techniques was written for a text on methods in pharmacology.

Significance to Biomedical Research and the Program of the Institute: The continuing interest in fluorescence methods is consistent with the tradition of leadership in this field shown by this institute. Many areas of biomedical research utilize fluorescence methods.

Proposed Course: Equipment will be evaluated as it becomes available. Some previous correlations of quantum yields of different compounds might be published as a contribution to resolving some of the controversy here.

We plan to evaluate various flow orienting devices such as those used in flow dichroism absorption studies, with a view towards using them in fluorescence studies.

Honors and Awards: None

Publications:

1. Chen, R.F., Fluorescence Spectroscopy for publication in Methods in Pharmacology, Vol. II, edited by C.F. Chignell, Appleton-Century-Crofts, N.Y., 1971, in press.

Serial No. NHLI-263
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Fluorescent Complexes of Proteins

Previous Serial Number: NHLI-184

Principal Investigator: Raymond F. Chen

Other Investigators: Dr. Roger M. McKinney
Center for Disease Control
U.S. Public Health Service
Savannah, Georgia 31402

Project Description:

Objectives: To determine the fluorescence parameters (quantum yields, lifetimes, spectra) of protein-dye complexes. Such complexes are of basic usefulness in immunofluorescence microscopy and physical biochemistry.

Methods Employed: Fluorescence measurements were used which have been described by this laboratory previously for determination of quantum yields, lifetimes, spectra, and polarization.

Major Findings: This year's work involved three major types of complexes:

1. The conjugate of lysozyme and DNS (1-dimethylamino-naphthalene-5-sulfonyl) was studied. It was found that this enzyme reacts with DNS-Cl to give a conjugate with only 1 DNS group per molecule of protein. The site of attachment was the epsilon-amino group of a lysine, as determined by chromatography of a hydrolyzate of the protein, and by comparison of amino acid analyzer assays of labeled and unlabeled proteins. The DNS group appeared to be lost from the protein over a few days, was in an exposed position as determined by the red-shifted position of the fluorescence peak, and by its freedom to rotate independently of the rest of the protein, as determined by fluorescence polarization. Current efforts are being made to identify the exact site of attachment of the dye. This is being done with tryptic digests of reduced, aminoethylated, carboxymethylated DNS-lysozyme; isolation of fluorescent peptides suggests a single site of attachment. The signficance of knowing where the dye is attached is that one can then

correlate the physical and fluorescence properties with the three dimensional structure obtained from X-ray crystallography.

2. Bilirubin-albumin conjugates were studied, using mainly bovine and human albumins, but also that from rabbit and sheep. The stoichiometry and strength of binding was studied by fluorescence quenching. Human albumin (HSA) and bovine albumin (BSA) bound 1 bilirubin very strongly, k_1 being 2×10^7 and 7×10^7 M^{-1} respectively. These constants have never been obtained accurately before. Energy transfer calculations based on the extent of fluorescence quenching show that the bilirubins are bound at least 25 angstroms from the tryptophans. Other data obtained include difference absorption data detailing the differences in properties of the first bound bilirubin compared with subsequently bound bilirubins. Also, data were obtained on the photostability, binding constants, and fluorescence of the bound bilirubins. A preliminary note to Science was rejected as being of too limited interest, but a more detailed manuscript is in preparation.

3. Gamma globulin labeled with fluorescein were studied in collaboration with Dr. McKinney, a pioneer in the use of such conjugates in immunofluorescence. The problem was to determine which isomer of fluorescein isothiocyanate, FITC I or FITC II, was to be accepted as a standard for such work. Dr. McKinney prepared and sent us samples of rabbit gamma globulin labeled to different extents with FITC I and FITC II. These dyes differ only in the position of the isothiocyanate group. We measured the fluorescence quantum yields. FITC I conjugates had an absolute quantum yield of 0.418 on excitation at 492 nm, while FITC II conjugates gave 0.391. Heavier labeling decreased the yields. More significantly, excitation at 366 nm which is used in immunofluorescence microscopy gave much more fluorescence with FITC I conjugates. This was found to be due to a higher extinction coefficient at this wavelength compared with FITC II conjugates. The quantum yields at 366 nm were much reduced in the FITC II conjugates compared with 492 nm excitation; this probably was due to impurities. The results will be written up by Dr. McKinney and are the first definitive evidence that FITC I is superior to FITC II for such work.

Significance to Bio-medical Research and the Program of the Institute: These results with enzymes, albumins, and gamma globulins are of fundamental interest and are consistent with the Institute's traditional efforts in basic and applied biomedical research.

Proposed Course: We plan continued efforts to identify the site of attachment of DNS on lysozyme, using sequencing techniques. We plan to finish up the work on bilirubin, especially the preparation of manuscripts. Possible future projects include investigation of flow-oriented dyed macromolecules, and the effects of denaturation on the spectra of various dyes on proteins.

Honors and Awards: None.

Publications:

Chen, Raymond F., Limited Reaction of Lysozyme with a Fluorescent Labeling Agent, Biochem. and Biophys. Res. Comm. 40, 1117-1124, 1970.

Serial No. NHLI-264
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Applications of Fluorescence in Biochemistry

Previous Serial Number: NHLI-182

Principal Investigator: Raymond F. Chen

Other Investigators: Dr. Thomas Wuntch and Dr. Elliot S. Vesell
Milton S. Hershey Medical Center
Pennsylvania State University
Hershey, Pennsylvania

Dr. Harvey Epstein and Dr. Alan N. Schechter
and Dr. Harvey Pollard
Laboratory of Chemical Biology, NIAMD

Project Description:

Objectives: Application of fluorescence techniques developed in this laboratory to problems of interest, thus testing and improving these techniques under conditions of actual use. Fluorescence methods sometimes are the only ways certain biophysical phenomena can be studied.

Methods Employed: The methods for following stopped flow kinetics of fluorescence changes in biological systems have been described from this laboratory previously, as have the methods for determining fluorescence yields, lifetimes, and spectra.

Major Findings: These different problems were studied:

1. Lactate dehydrogenase isoenzymes were studied at high concentrations in collaboration with Drs. Wuntch and Vesell. Some results were published previously, showing that the different isoenzymes had different properties at high concentration such as occurs physiologically, as compared with the artificially low concentrations used in traditional enzyme assays. According to a theory of N.O. Kaplan, the raison d'etre for isoenzymes is that they are inhibited to different extents, thus having certain advantages in aerobic and anaerobic tissues. For example, lactate dehydrogenase in skeletal muscle is apparently more inhibited by pyruvate than is heart muscle lactate dehydrogenase. Under anaerobic conditions such as muscular exertion, therefore, pyruvate

accumulates and its carboxylation to oxalacetate is favored, thus permitting an alternate metabolic path. We found, however, that the inhibition is not very significant at high enzyme concentrations, and that the presence of other proteins reduces what little inhibition there is. These experiments tend to reduce the likelihood that the Kaplan theory explains the existence of isoenzymes.

2. The folding of staphylococcal nuclease was studied with Drs. Epstein and Schechter. Previously, we reported (Schechter, Chen, and Anfinsen, Science 167:886, 1970) that fluorescense stopped flow data showed that nuclease refolding could be described by two first order processes with half times of 55 and 350 milliseconds. The experiment follows the change in fluorescence of the protein in acid, where it has low emission and is unfolded, when the solution is neutralized and the enzyme takes up its native conformation where the single tryptophan is buried and has high quantum yield. We have extended these studies by observing the effect of different ionic strengths and temperatures. It was discovered that the fast process was temperature independent, whereas the slow process decreased to half-times of 750 msec. at 13°C and increased to 180 msec. at 38°C. From the known enthalpic and entropic changes of various folding processes, it was inferred that the rapid part of the refolding was probably helix formation in the region of the tryptophan. This was followed by the slow process, which was probably the formation of hydrophobic bonds. The thermodynamic parameters were calculated from the van't Hoff and Arrhenius relations. A paper, "The Folding of Staphylococcal Nuclease: Kinetic Studies of Two Processes in Acid Renaturation" by H.F. Epstein, A.N. Schechter, R.F. Chen, and C.B. Anfinsen has been submitted recently to the J. of Molecular Biology.

3. Low density lipoprotein (LDL) was studied in collaboration with Dr. Harvey Pollard. The emission peak of LDL was at 325 nm which is very low, and indicates a hydrophobic environment for the emitting tryptophans. On extraction of the neutral lipids, the apo-LDL emitted maximally at 332 nm. Yet another emission spectrum was obtained when the phospholipids were removed from the apo-LDL. Correlations with the change in amount of alpha-helix were made from simultaneous circular dichroism data. The results show that removal of neutral lipids decrease the amount of helix, and removal of the phospholipids increases the amount again; however, the protein is now refolding in a way which is not characteristic of the native structure.

4. Fluorescence quenching by mercuric ion interaction with proteins and aromatic amino acids was studied. It was found

that mercuric ion quenched tryptophan, tyrosine, and phenyalanine fluorescence. In proteins, mercuric ion invariably quenched, and a major mechanism appears to be the transfer of energy from tryptophan to the tail end of the Hg-sulfhydryl absorption band. Critical transfer distances were calculated for ovalbumin-Hg complexes and for model systems. The quenching by silver ion also appeared to be due to energy transfer. Mercuric ion interaction with indoles on the other hand results in quenching due to the formation of complexes which have different absorption spectra. Mercuric ion may be a useful probe to assay distances between sulfhydryl groups and tryptophan. This is also a good way to determine the number of sulfhydryl groups.

5. The question of exciplex formation in indole compounds was studied. R. Lumry's group postulated that indoles, including tryptophan in proteins, formed excited state complexes with solvent molecules to give "exciplexes".They based their theory on the supposed finding that the emission spectrum of indoles was influenced by the presence of polar solvent molecules which the absorption spectrum was not. Van Duuren had reported the same thing. On reexamining the evidence for exciplex formation, we found that cyclyhexane solution of indole containing 1% ethanol or butanol had significantly different absorption spectra compared with the pure cyclohexane solutions. Other indole compounds showed similar changes. N-Methylindole showed neither fluorescence nor absorption changes in the presence of 1-2% alcohol. Solvation involving the N-proton occurs in the ground state. Thus, there is no experimental support for the idea of exciplex formation in the indole series. A short communication detailing these findings has been submitted to Archives of Biochemistry and Biophysics.

Significance to Biomedical Reseach and the Program of the Institute: The various projects listed above involve novel techniques (stopped-flow kinetics of protein renaturation) or new phenomena (e.g., mercuric ion quenching of protein fluorescence). The results add to the store of basic knowledge of these systems and demonstrate the usefulness of new fluorescence methods.

Proposed course: Data have already been obtained on the kinetics of acid-to-neutrality renaturation of albumin, of urea denaturation of asparaginase and nuclease, as well as renaturation by dilution of urea solutions. These projects can be completed, and will test the variable-ratio stopped-flow mixer. The results of silver ion quenching of fluorescence are ready for reporting. We intend to continue applying fluorescence techniques to questions of protein shape changes and binding problems.

Honors and Awards: None

Publications:

1. Wuntch, T., Chen, R.F., and E.S. Vesell, Lactate Dehydrogenase Isozymes: Further Kinetic Studies at High Enzyme Concentrations, Science 169, 480-481, 1970.

2. Chen, R.F., Fluorescence Quenching Due to Mercuric Ion Interaction with Aromatic Amino Acids and Proteins, Arch. Biochem. Biophys., in press.

Abstracts:

1. Pollard, H.B., and R.F. Chen, Differential Influence of Neutral- and Phospho-Lipids on the Protein Conformation of Human Low Density Lipoprotein. For presentation at the American Society of Biological Chemists meeting, San Francisco, June, 1971.

2. Schechter, A.N., Epstein, H.F., Chen, R.F., and Anfinsen, C.B., Kinetics of Folding of Staphylococcal Nuclease, idem.

Serial No. NHLI-265
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Countercurrent Chromatography: Liquid-Liquid Partition Chromatography without Solid Support

Previous Serial Number: NHLI-181

Principal Investigator: Yoichiro Ito

Other Investigator: Robert L. Bowman

Cooperating Units: None

Project Description:

Objectives: Development of a new technique for liquid-liquid partition chromatography without a solid support.

Methods Employed: The method employs a vertical helical tube in a centrifugal field. The tube makes one counter rotation per revolution thus eliminating the need for rotating seals. When the tube is filled with the stationary phase and the moving phase is introduced from the entrance of the tube, a hydrodynamic phase equilibrium is quickly reached and oscillating alternating segments of the two phases are formed throughout the tube. Further feeding of the moving phase results in the displacement of the moving phase only, leaving a given volume of the stationary phase in each coil unit. Consequently, a solute introduced into the tube is subjected to a rapid partition process between the oscillating segments of the two phases and finally eluted out from the exit end of the tube.

A test system was made by modifying a conventional centrifuge. The motor shaft was extended through the top-end bearing of the stationary tube mounted to the motor housing and then connected to a rotating tube that fits freely over the stationary tube with the bottom bearing. A pair of arms extending from the rotating tube holds coil holders with bearings. A toothed belt is then inserted between a pair of toothed pullies, one fixed to the bottom of the stationary tube and the other at the bottom of the coil holder to produce the desired planetary motion to the holder. The column is made of Teflon tubing either by winding the tube onto the coil holder or by arranging multiple column units interconnected in a series around the holder. The leading tubes are passed through the center hole at the top of the holder and then supported at the level of 25 cm. above the

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center of the apparatus. The liquids are fed with a metering pump and the effluent is monitered through a flow cell at 280 nm.

Analytical and preparative potentiality of the method was evaluated on DNP amino acid separation by using a two phase system composed of chloroform, glacial acetic acid and 0.1 N HCl (2:2:1).

Major Findings: Analytical capability of the method was examined on a column prepared from a 100 m section of 0.3mm I.D. tubing coiled onto a 5mm diameter core. At 550 rpm with a 30.7cm radius of revolution and at a flow rate of 2.4 ml per hour, 10 µl of a nine DNP amino acid mixture is eluted out within 10 hours at an effeciency ranging from 10,000 to 3,000 T.P. (theoretical plates).

The preparative column is prepared from a 90m piece of 1.2mm I.D. tubing coiled onto a rod measuring 0.9cm in diameter. At 520 rpm with an 8.6cm radius of revolution and at a flow rate of 24 ml per hour, 1 ml of the DNP amino acid sample is eluted out within 13 hours, yielding an efficiency ranging between 4,000 and 1,000 T.P.

The method is reproducible with minimum carry-over of the stationary phase. In addition, the entire procedure including the column preparation is simple and easy for even an unskilled person to follow.

Preliminary experiments to separate dansyl-peptides on various phase systems have shown promising results.

Significance to Bio-medical Research and the Program of the Institute: The support free liquid-liquid partition technique is an ideal method of separation since it eliminates the complications arising from a solid support. When the method is refined to cover both analytical and preparative use, it will replace the conventional liquid-liquid partition techniques now used in biomedical research.

Proposed course: The future plan may be

1. Further improvement of the apparatus.
2. Investigations on various factors involved in the method.
3. New applications of the apparatus.

Honors and Awards: None.

Publications:

1. Ito, Y. and Bowman, R.L.: Countercurrent Chromatography with Flow-Through Coil Planet Centrifuge. Science, in preparation.

2. Ito, Y. and Bowman, R.L.: Countercurrent Chromatography. Anal. Chem., in preparation.

Serial No. NHLI-266
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Countercurrent Chromatography: Liquid-Liquid Partition Chromatography without Solid Support, (Part II)

Previous Serial Number: NHLI-181

Principal Investigator: Yoichiro Ito

Other Investigator: Robert L. Bowman

Cooperating Units: None

Project Description:

Objectives: Development of Gyration Locular Countercurrent Chromatography (Gyration L.CCC).

Methods Employed: The apparatus for Gyration L.CCC requires a well balanced design. Our prototype consists of a pair of perpendicular rotary shafts held 9 inches apart. Each end (top and bottom) of the shafts is equipped with a disc carrying an eccentrically mounted bearing. A pair of horizontal arms, one bridging the top bearings and the other the bottom bearings, hold a cylindrical column holder vertically at the center of the arms. A pair of toothed pullies each fixed near the middle portion of the rotary shaft is synchronously driven by a toothed belt connected to another pully fixed to the motor shaft. Counter-balancing of the system is well established by applying proper weights symmetrically on the rotary shafts. The gyration speed is regulated by a speed control unit and continuously adjustable up to 1.500 rpm.

The locular column is prepared by placing centrally perforated Teflon partitions across the diameter of a Teflon tube. Partition pieces in a variety of sizes and configurations are used to examine the performance in separation especially in relation to the column efficiency and the carry-over of the stationary phase.

The capability of the method is examined on DNP amino acid separation with a two phase system composed of chloroform, glacial acetic acid and 0.1 N HCl (2:2:1).

Major Findings: A simple mathematical analysis on the motion of the two phases in a gyrating locule indicates that the interfacial shape is circular on cross section and parabolic on vertical section. Further the centrifugal force acting on the liquids forms a gradient along the line through the center of gyration to an arbitrary point of the locule, suggesting that the lighter phase is always subjected to less centrifugal force and tends to form emulsification and carry-over of the heavier phase. Thus, the radius of gyration should be much greater than the locule diameter, if the carry-over of the stationary phase is to be eliminated.

The nature of the partition hold is directly related to the carry-over phenomenon. When the exit hold of the locule is made small and wettable to the moving phase, the carry-over is largely eliminated even at a high gyration speed. Also, a threaded partition hole prevents the carry-over if the direction of the gyration is chosen to hold down the droplets of the stationary phase in the locule.

Tendency of the longitudinal diffusion of the solutes through the column was examined by stopping the flow at various intervals and measuring the resultant broadening at the eluted solute peak. Vibration of the apparatus was found to be critical whereas, in a well balanced system and on a column with small partition holes, the longitudinal diffusion becomes negligible.

The column efficiency generally increases with column length, locule number, a high gyration speed and a slow flow rate. The efficiency also exhibits a tendency to decrease with increased retention time of the solutes. At a flow rate of 12 ml per hour and 800 rpm with a 2.5 cm radius of gyration, a column with 1,600 locules, each 4mm I.D., 1.7mm long, and a glass bead (0.5mm I.D., 1mm long) embedded at the partition hole, yields an efficiency ranging from 1,600 T.P. (partition coefficient: 3.8) to 740 T.P. (partition coefficient: 0.18) or 100% to 40% partition efficiency per locule.

Significance to Bio-medical Research and the Program of the Institute: The support-free liquid-liquid partition technique is an ideal method of separation since it eliminates the complications arising from a solid support. When the method is refined to cover both analytical and preparative use, it will replace the conventional liquid-liquid partition techniques now used in bio-medical research.

Proposed Course: The future plans may be summarized as follows:

1. Refinement of the apparatus to reduce vibration at a higher rate of gyration.

2. Further investigations on various factors involved.

3. Application for separation of macromolecules and particulates on polymer phase systems.

Honors and Awards: None

Publications: None.

Serial No. NHLI-267
Laboratory of Technical Development
Section on Pulmonary and Cardiac
Assist Devices
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of the Spiral Coil Membrane Blood Oxygenator and Systems for Long-term Temporary Support of Pulmonary and Cardiovascular Systems.

Previous Serial No.: NHLI-178(c)

Principal Investigators: Theodor Kolobow

Other Investigators: Roger Spragg, Joseph Pierce, Gerald Vurek, Warren Zapol, and Robert L. Bowman

Cooperating Units: Laboratory of Kidney and Electrolyte Metabolism
Becton-Dickinson Research Center, Raleith, N.C.
Applied Science Division, Litton Industries

Project Description:

Objectives:

1. To optimize the design of the spiral coil membrane artificial lung and to apply the artificial lung to clinical support of respiratory and cardiac insufficiency. We will demonstrate the superiority of membrane blood gas exchange in perfusion systems by long term animal studies designed to test the perfusion apparatus and explore the clinical limitations of this method. A clinically acceptable system will be constructed from highest quality minimum defect membrane that can be developed and manufactured.

2. Current extracorporeal bypass technique uses heparin as the anticoagulant. Long term perfusions similarly must use heparin with the ever present danger of serious bleeding from open wounds, or spontaneous hemorrhage. We have explored methods of reducing systemic heparin requirement after the first day of high heparin infusion to reduce or eliminate wound bleeding, or spontaneous bleeding.

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Methods Employed and Major Findings:

1. The membrane artificial lung. Membrane artificial lungs with blood oxygenating capacity up to 5-l/min have been built by the Applied Science Division of Litton Industries. Production techniques have included test procedures to assure absence of leaks and high quality performance. The most important factor to the delivery of leak-proof membrane lungs remains the continuous availability of dependable pin-hold free silicone rubber membrane.

A. Animal Research.

1. Extracorporeal respiratory blood gas exchange with the membrane lung has been performed in unanesthetized lambs for up to 16 days. At autopsy, the lungs and other internal organs of the animal perfused for sixteen days were found to be normal.

2. Total cardiopulmonary bypass by peripheral cannulation using the membrane lung was carried out in unanesthetized lambs with induced ventricular fibrillation. Normal blood respiratory gas tensions can readily be maintained. At high bypass flows the central aortic pressure in lambs approaches normal, and reaches normal at bypass flows equal to control cardiac output.

3. Systemic heparin requirement can safely be reduced after the first 24 hours of high systemic heparinization to yield a modified Lee White clotting time of 2-8 hours. We have seen tolerance develop after continuous administration of heparin.

4. Wound bleeding using present improved bypass cannulation techniques and lowered heparin infusion is virtually eliminated. We have found that interference in peripheral venous and arterial circulation from peripheral cannulation uniformly creates serious problems in wound bleeding and arterial vascular insufficiency. We now routinely restore circulation distal to our cutdown site.

B. Clinical Applications: This laboratory has received frequent requests to provide emergency extracorporeal respiratory blood gas exchange in patients with acute respiratory failure. Requests have come from Michigan, Pennsylvania, Virginia and locally. Logistical problems have been a major cause in our reluctance to overextend cur limited manpower resources. We have been ready on several occasions

to actually provide bypass assist if that proved to be life saving. However, no actual perfusions for respiratory insufficiency have been carried out during the past year.

2. Large bore non-kinking blood cannula. Stainless steel spring wire was coated with elastomeric polyurethane to provide large bore, non-kinking cannula and non-collapsible blood conduits. We have found these cannulae superior to all blood conduits devised so far, and being particularly responsible for the success of our prolonged animal experiments. We feel that its availability will be of immeasurable help to the research community. Extracorporeal blood flow as high as 160-180 cc/kg/min has been obtained routinely in some of our animal studies. The availability of these special catheters is still limited. Ethicon, Inc. and Benedum Instruments are at present supplying these catheters in small quantities.

3. Atraumatic extracorporeal blood pump. The fiberglass reinforced blood pump chamber has exhibited wear characteristics well beyond our expectations. We are presently still using a single fiberglass reinforced pump chamber that has cumulatively been used for over 6 months without appreciable wear. We believe that pump chamber wear is significantly reduced by maintaining a non-occlusive pumping mode; we also believe that this has contributed significantly to reduced blood trauma.

Significance to Biomedical Research and the Program of the Institute: We have established that it is practucal to maintain pulmonary function in animals with a membrane artificial lung long enough to presumably allow recovery of damaged pulmonary or cardiac function. In a few clinical instances nothing has happened to reduce our confidence, but so far complete recovery after clinical application has not occurred. Lack of damage in animals and the few clinical applications suggest that adequate gas exchange can be maintained for weeks if necessary and that death due to inability of damaged lungs to function can be forestalled long enough for reparative process to reestablish normal function.

In maintaining alert animals with a fibrillating heart it suggests that bypass oxygenation can relieve the heart from the burden of maintaining pulmonary circulation and possibly contribute to survival from myocardial infarction.

The improvement in perfusion technology occasioned by developments in catheter, pump chambers, and instrumentation for maintaining optimum phusiological conditions emphasized the need for continued development of instruments for optimum use of modern technology in medical therapy.

Proposed Course:

1. Clinical studies in long-term respiratory assistance with an extracorporeal membrane lung will be continued. Clinical facilities are being set up at a central place to permit prompt initiation of membrane lung assist for respiratory failure. These facilities can be equally suitable to provide cardiac assist in patients in need of temporary circulatory support.

2. We will continue our studies to evaluate long-term total cardio-pulmonary assist with the extracorporeal membrane lung perfusion system in unanesthetized animals.

3. Our program in long-term extracorporeal respiratory blood gas exchange and cardiac assist will be extended. It will include study of factors that will make short term perfusions clinically safe; it will explore methods that will allow perfusion to be performed for as long as one month. High priority will be given to the use of a "zero defect" membrane lung, and the incorporation of state of the art hypothrombogenic membrane surfaces.

4. Our contract with the Becton-Dickinson Research Center will be extended to explore methods for the manufacture of "zero defect" spiral membrane lungs. Main emphasis will lie in the production of reinforced high quality, defect free silicone rubber membrane. At the same time, they will supply us with membrane lungs of differing capacity and assembly for laboratory use.

Honors and Awards: None

Publications:

1. Kolobow, T.; Spragg, R.G.; Pierce, J.E.; and Zapol, W.M.: Extended Term (to 16 days) Partial Extracorporeal Blood Gas Exchange with the Spiral Membrane Lung in Unanesthetized Lambs, Proceedings of the American Society for Artificial Internal Organs, Vol XVII, in press.

2. Kolobow, T. and Zapol, W.M.: Partial and Total Extra-Corporeal Respiratory Gas Exchange with the Spiral Membrane Lung, Advances in Cardiology, in Press.

3. Zapol, W.M.; Kolobow, T.; Koppman, J.; and Pierce, J.E.: Response of Ductus Arteriosus and Pulmonary Flood Flow to Blood Oxygen Tension in Immersed Lamb Fetuses Perfused through an Artificial Placenta, J. Thoracic & Cardiovascular Surgery,

Serial No. NHLI-268
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Dual-Frequency Ultrasonic Flowmeter

Previous Serial Number: NHLI-190

Principal Investigator: Frank W. Noble

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: To modify and evaluate an ultrasonic flowmeter for measurement of blood flow.

Methods Employed: The present flowmeter system was described in Individual Project Reports NHI-200, NHI-330, and NHLI-190. We have continued to investigate methods for reducing zero drift and improving accuracy of flow indications.

Major Findings: Two major deficiencies of this and all other cw phase measuring systems are artefacts due to reflected waves and drift in both zero and slope due to the fact that time measuring systems are affected by change in the sound speed in the liquid. A new system has been conceived in which frequency is automatically varied to force a constant wavelength in the cell. Measurement of the frequency difference between upstream and downstream sound propagation yields flow speed independent of sound speed, and average frequency yields sound speed independent of flow speed. In addition, it is expected that artefacts due to reflected waves will largely disappear because the number of wavelengths in the cell path is held constant.

Signficance to Bio-medical Research and the Program of the Institute: Accurate, dependable flow measurement in vessels of varying size and location continues to be a problem. We favor the ultrasonic system because the transducers are simple and small and because it is possible to telemeter through intact skin.

Proposed Course: The new constant-phase system will be built and tested in vitro. A comparison will then be made

against the system now in being, and the better method will be further refined.

Honors and Awards: None

Publications: None

Serial No. NHLI-269
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Peltier-Seebeck Equilibrator

Previous Serial Number: None

Principal Investigator: Frank W. Noble

Other Investigators: Robert L. Bowman
Peter Carmeci

Cooperating Units: None

Project Description:

Objectives: We wish to measure the rate of heat generated within a small (less than 4 mm^3) cell without allowing the cell temperature to rise above a desired ambient. If sufficient sensitivity can be obtained, it may be possible to monitor the growth rate of bacteria at constant temperature. Other possibilities include the study of thermal properties of chemical reactions at constant temperature.

Methods Employed: Since the advent of commercially available Bismuth Telluride Alloys, it has become practical to produce miniature refrigerators using the Peltier cooling effect. These devices can also sense temperature differences by means of the Seebeck effect. We have designed and built a device which senses temperature difference during half of a cycle and restores temperature equilibrium during the other half of the cycle. The Peltier current required to maintain temperature equilibrium is a measure of the heat rate output of the cell contents, while the temperature remains essentially constant.

Major Findings: Known heat rate inputs have been produced by passing measured currents through a miniature resistor located within the cell. Under open-loop conditions, it has been possible to detect heat rates of 25 microwatts, corresponding to the heat output of about one million bacteria.

Significance to Bio-Medical Research and the Program of the Institute: If it were possible to record the growth rate of bacteria continuously and without attention, the study of the effects of temperature, drugs, nutrients, etc. would be immensely facilitated.

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Proposed Course: We intend to increase the thermal sensitivity of the instrument and reduce the physical size of the cell to the greatest possible extent. Tests will be made using live bacteria in addition to the resistor heat generator, so that the cell thermal sensitivity may be calibrated with the bacteria in place. Clock-type chemical reactions will be observed to obtain their time course as well as their total heat output.

Honors and Awards: None

Publications: None

Serial No. NHLI-270
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An Automated Method for Rapid Bacteriological Assay

Previous Serial No.:

Principal Investigator: Robert L. Bowman

Other Investigators: Peter Carmeci
Gerald G. Vurek
Nelson Braslow

Cooperating Units: Clinical Chemistry Lab.
Department of Laboratory Medicine
University of Minnesota Hospital

Laboratory of Infectious Diseases, NIAID

Project Description:

Objectives: The objectives of this project are to develop the techniques and methods necessary to facilitate the utilization of the capillary tube scanner for clinical and research application. At the present time efforts have been expended in the following five areas of endeavor.

A. Developing methods and teqhniques for automating antibiotic sensitivity tests.

B. Developing methods of pulse height discrimination for segregating types of colonies during incubation.

C. Developing methods for early detection of Mycoplasma (Pneumonia).

D. Development of methods for detecting the growth characteristics and effects of antimetabolites on myeloma and other neoplastic cell types.

E. Development of a method for isolating and monitoring the growth of extremely small quantities of bacteria.

A. Antibiotic Sensitivity.

The efforts to date have been oriented towards developing an inexpensive and readily automated

method for performing antibiotic sensitivity tests using the Colony Counter system developed here. The approach has been to replace the capillary tubes with a single plastic plate containing 14 channels that will accept a mixture of agar and organisms. Each channel of the plate contains a strip of antibiotic impregnated filter paper. This paper is bonded to the side of the channel so that the light path is not obstructed. The entire plate is covered with an adhesive backed mylar film. The entire plate can readily be prepared and stored prior to injection of an agar-bacteria solution. To date a plate has been designed and the technique is undergoing tests by Dr. Blume at the University of Minnesota. Subsequent work will be aimed at automated methods for preparing and filling the plates. Commercial prototype instruments provided by Aminco are being used in this project. A patent has been granted for the bacterial counter.

B. Pulse height discrimination for bacteris has been investigated using manual techniques. This has shown that further investigation would be fruitful, in that we demonstrated the growth rates of E. Coli, Strep, Mycoplasma and Myeloma cells can be readily characterized and subtle changes in growth can be distinguished.

C. A number of attempts at trying to detect mycoplasma (Pneumonia) have been tried using the capillary scanner. Because of the long time required to incubate these mycoplasma it would be very beneficial to be able to detect and count them at an early stage in their development. The usual methods of mixing organisms into agar and filling the capillaries is not conducive to mycoplasma growth. The characteristic of micoplasma to settle onto the walls of the glass capillary while in a broth was exploited. Preliminary results have shown that by filling the capillaries with a mixture of broth and organisms and allowing them to settle for a day or so, that they can be detected and counted in about 48 hours. Further testing and development of an optimum technique is presently underway.

D. Myeloma growth characteristics. Preliminary investigations in detecting the growth curves of Myeloma cells and the effects of chemotherapeutic antimetabolites has been successful. It has been demonstrated that cell sensitivity to specific

metabolites at known concentrations can be determined readily within 13 hours. Further investigations will attempt to determine effective doses of specific antimetabolites for Myeloma and other types of neoplastic cells.

E. Efforts have been expended in the preliminary investigation and development of a method for aligning bacterial cells in agar in a single line about the peripheral surface of an optically clear planchet. The bacteria are aligned by centrifugation in the planchet and their growth and susceptability to antibiotic agents can readily be monitored optically or by scattered light. Bacterial suspensions of 100/ml have been centrifuged at 6000 RPN and grown in a line about the periphery of a 1-1/2" diameter planchet. It has been demonstrated that the growth of an individual colony can readily be maintained. Efforts to date have been expended in designing and developing techniques for centrifugation. Future efforts will be directed towards greater dilutions of bacterial suspensions and design of smaller planchets.

Significance to Biomedical Research and the Program of the Institute: The utilization of light scattering techniques in bacteriology provides (a) means of detection very early in the growth of colonies and (b) methods that are readily adaptable to automation and require extremely small samples.

Proposed Course:

A. Continued application to fully automate the antibiotic sensitivity testing procedures.

B. Developing hardware for pulse height analysis and investigating its utilization for segregation of two or more organisms by their growth rates.

C. Developing techniques for detecting and measuring mycoplasma (Pneumonia), in cooperation with Dr. Brunner, NIAID.

D. Investigation of effects of antimetabolites on Myeloma and other neoplastic cell types.

E. Further development of methods for centrifugation and monitoring of cells.

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Honors and Awards: None

Publications: None

Serial No. NHLI-271
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Blood Flow Measurement Using Nuclear Magnetic Resonance Techniques

Previous Serial Number: NHLI-180

Principal Investigators: Vsevelod Kudravcev, Robert L. Bowman

Other Investigators: Anthony Sances, Jr., Ph.D.
Joseph H. Battocletti, Ph.D.

Cooperating Units: Medical College of Wisconsin
Milwaukee, Wisconsin

Project Description:

Objectives: Nuclear magnetic resonance phenomonae provide a unique approach to physiological measurements because protons can be stimulated to absorb or emit electromagnetic radiation that can pass freely through all tissues. Protons have a gyromagnetic moment that can be influenced by magnetic and electromagnetic fields to cause resonance at a specific frequency. This resonance and nuclear orientation decays with a time constant of almost half a second so that the NMR system can mark protons so that they can be detected for several seconds. Several flow measuring systems have been investigated and reported previously but present attention is directed to new systems that permit highly specific localization of the marked protons with the expectation that a three dimensional map of cerebral perfusion parameters could be produced by entirely non-invasive observations. The presumption being that such observation could be applied to discovering what part of the population is at greater risk of having a cerebral vascular accident. In addition such observation might provide a guide to therapy by indicating progression or resolution.

Present observations have been limited to model systems where it has been established that we can, by application of a large magnetic field for a second or two to flowing blood, render this blood specifically susceptible to a second electromagnetic field that can erase the magnetic marker. When the second field is spaced along the course of the blood flow only that blood that was in the sphere of influence of the second field will have the signal erased. When the erasing field is pulsed the blood passing thru that region will have a modulated marker that can be detected in the blood draining that region.

The fact that the erasing field can be localized in space and that the head can be positioned in that space suggests that a specific region can be measured and changes in flow produced by CO_2 inhalation evaluated.

A proposed configuration for the measurement of cerebral blood flow includes a polarizing magnet in the thoracic region, a depolarizing scheme steerable throughout the head region and a detector coil in the neck region. Relative transit times from selected regions in the head to the detector compared between right and left sides can be used to establish circulatory abnormalities. The physical size limitations of the patient and the equipment as well as the time restrictions established by typical flow patterns require that the nuclear magnetic resonance detection system have great sensitivity and versatility. The work performed during the past year has been aimed at improving the detection scheme.

Major Findings: Several new methods of signal detection developed near the end of the 1970 report period have been confirmed to have sufficient promise that the major activity this year was devoted to reconstructing the equipment to permit the use of those new signal detection systems and apply them to structures as large as the head and neck.

The need for highly sensitive detection systems is based on the fact that most protons lose the marker with a time constant of 0.4 seconds while the transit time thru the head is on the order of 7 seconds. We have decided to determine the volume resolution that these times will permit in corss perfusion systems that are being run for us under a contract with the Medical College of Wisconsin. The cooperation with the Medical College (Dr. Sances) provides a team that includes a NMR engineer and the physiological and surgical faculties of the medical school where animal experiments can be performed and correlated with cerebral radioangiography and radio isotope methods.

In this first year the contractors have confirmed our tag detect system in monkey limbs and the ability to detect the tagged flow when flow was only one thousandth of the volume of the tissue in the detector head.

Design criteria have been established for the equipment to be used at the Medical College of Wisconsin which will be based on conversion of commercially available NMR system used to measure flow in pipes sold by the Badger Meter Company. It is anticipated that a commercial source of this rather specialized gear can be developed by further cooperation with this company.

Significance to Biomedical Research and the Program of the Institute: These non-invasive techniques hold promise to discover those individuals who are at risk due to vascular disease predisposing to stroke. If sufficient sensitivity and spatial discrimination can be obtained the method may be applicable to evaluate therapy by measuring progression or improvement. The same technique or a modification has potential for measuring myocardial blood flow with similar limitations and potentials.

Proposed Course: This laboratory will assemble the new system that will permit the refinement of the electronic signal modulation and detection at low field strengths that optimize conditions for detecting the weak signal in the neck.

The apparatus built here will permit a wide selection of modulation and detecting methods to be evaluated in the special physiological circumstances that prevent clear predictions of the best method to be make.

The contract with the Medical College of Wisconsin is being renewed to permit observations on animal and humans to be made with a selected group of apparatus adaptable from commercially available pipe line flow metering equipment. Cross circulation experiments to determine what proportion of labeled protons excapt from the circulation and what their escape does to limit the measurements. Some newer mechanical modulation that may provide additional specificity has already been suggested to increase the versatility of the techniques.

Honors and Awards: None

Publications:

Battocletti, J.H.; Bowman, R.L.; Kudravcev, V.; Sances, A.; Jr.: Larson, S.J.; Lineham, J.H.; Halback, R.E.; Antonich, F.J.; Genthe, W.K.; and Evans, S.M.: An off-resonance non-intrusive magnetic method for measurement of blood flow, J. for the American Adv. of Med. Instru., In Press.

Serial No. NHLI-272
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: An Investigation and Development of Methods for the Study of the Mechanism of Enzyme Action and Function in Cellular Systems and in Solution

Previous Serial No.: NHLI-193

Principal Investigator: Robert L. Berger

Other Investigators: N.O. Kaplan, J. Everse, L. Bernstein, R.R. Fisher, J.W. Roughton, M. Marini, A.A. Benson, E. Antonini, E. Baird Hastings, J. Massergian and L. Rossi-Bernardi

Cooperating Units: BEI-DRS, LPB-NIAMD; Institute of Chemical Biology, University of Milan; Institute of Molecular Biology, University of Rome; School of Medicine, Northwestern University; Department of Chemistry, Department of Medicine, Scripps Institution of Oceanography; University of California at San Diego; Jet Propulsion Laboratory; American Instrument Company, Silver Spring, Maryland; Department of Engineering Mechanics, Penn State University

Project Description:

Objectives: The objectives of this project are to investigate and develop new instrumentation methods, data handling techniques, and theoretical treatments for the physiochemical s u y of the thermocynamics, rates, and mechanisms of enzyme action in solutions and in the intact membrane. In particular to study, in collaboration with other laboratories, the mechanism of action of enzymes of the cardiovascular system and where appropriate application is indicated, develop clinical analytical methods using these techniques.

Methods Employed: The methods used in the investigation of mechanisms of enzyme action are those of pre-steady chemical kinetics, and thermodynamics. Measurements of the appropriate parameters are made by building the necessary equipment to mix solutions rapidly and follow the course of the resulting chemical reactions by optical, thermal, glass electrode. etc. In general, equipment is not available either in the literature or commercially,

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for investigations in this area. Such apparatus is conceived and designed in this laboratory, together with consultants, construction being carried out wherever most appropriate, i.e., in our shops or by commercial firms, special university facilities, or at the several special research laboratories such as the Jet Propulsion Laboratory. A special effort is then made to have commercial versions of the equipment available to the public. In pursuing these investigations, a wide variety of physical parameters must be studied which leads to the need for an understanding of the underlying physical theory governing the reactions and expert consultants and collaborators are brought in to assist in the design, analysis, and evaluation of the equipment, particularly as it applies to certain specific enzyme systems under investigation.

Major Findings: This year has been spent as a visiting scientist in the Department of Chemistry and the School of Medicine, University of California at San Diego. Much of the equipment that has been developed for the study of the mechanism of enzyme action has been brought here. The equipment, its area of usefulness and the interpretation of the data it provides, has been introduced to the faculty and students. The work may be divided into that being done with medically-oriented groups, enzymologists, physical biochemists, and analytical clinical chemistry. Of particular interest is the fact that since all of these people are using the equipment, they have been interacting while so doing which in itself has been most fruitful.

The use of the stopped-flow apparatus for the study of biochemical reactions has been extended to include fluorescence and polarization detection methods. By attaching Glan Prisms from the Aminco-Bowman spectrofluorometer, a very simple absorption or fluorescence polarization system can be set up. Three systems have been extensively investigated during the past year.

1. Investigation of the Mechanism of Action of Heart and Muscle Type Lactate Dehydrogenase: During our investigations into the chemical and kinetic mechanisms of various pyridine nucleotide linked dehydrogenases we studied the binding of 3-acetylpyridine-DPN+ (AcPyDPN+) to the lactate dehydrogenases of chicken heart and skeletal muscle. Since the binding of the coenzyme to the enzymes is extremely fast, we used an Aminco-Berger stopped-flow apparatus for these studies. The enzymes are tetramers, and bind four moles of coenzyme per mole of enzyme. Our results show, that the binding constants for each of the four coenzyme molecules are identical, suggesting that the four subunits act independent of each other with respect to the binding of the coenzyme.

The binding may be followed by the decrease in the protein fluorescence, as well as by the resulting enhancement of the coenzyme fluorescence. Figure 1 shows the decrease of the protein fluorescence as a result of the binding of four moles of AcPyDPN+ per mole of enzyme. The analysis of the observed rate indicates that the reaction is bimolecular, with an observed rate constant of 7.95×10^{7} liters mole $^{-1}$ sec^{-1}. Figure 2 shows the increase in the coenzyme fluorescence which is observed under the same conditions. The analysis indicates that the rate represents a unimolecular reaction, with an observed rate constant of 95 sec^{-1}. These results indicate that the rate represents a unimolecular reaction with an observed rate constant of 95 sec^{-1}. These results indicate that rollowing the binding of the coenzyme a rearrangement takes place in the complex, which is about a factor 3 slower than the actual binding itself. Such observations are of extreme importance for a proper understanding of the kinetic mechanism of an enzyme.

DNP+ in alkaline solutions reacts with pyruvate (and other nucleophillic reagents) to form adducts. The formation of the DPN-pyruvate adduct involves a nucleophillic attack by pyruvate at the 4-position of the nicotinamide ring, and a nucleophilic attack by the amide nitrogen at the carbonyl group of the pyruvate. Both muclrophilic reactions are base catalyzed. In order to ascertain in what order the two reactions occur, studies were performed with the use of the Aminco-Morrow stopped-flow apparatus. The reaction may be followed by measuring the increase in fluorescence (the product is fluorescent, whereas the reactants are not), as well as by measuring the increase in absorption at 340 mμ . Figure 3 shows that the absorption at 340 mμ increases immediately, whereas the increase in fluorescense shows a considerable lagtime. These results were interpreted to indicate that the first reaction involves the attack by pyruvate at the 4-position of the nicotinamide ring. This results in the formation of a dihydropyridine ring (see Figure 4), which is known to absorb at 340 mμ . The formation of the fluorescent compound is thus the result of the ring closure, by the attack of the amide nitrogen at the carbonyl carbon of pyruvate.

The elucidation of the sequence of the two reactions was of importance in the understanding of the chemical mechanism of lactate dehydrogenase.

H-type lactate dehydrogenases are inhibited by high levels of pyruvate during enzymatic assays. This inhibition is a result of the formation of a ternary complex, consisting of enzyme, DPN+ and pyruvate. The question thus arises whether this ternary complex may also be formed under physiological conditions, in which the enzyme concentration is estimated to be or the order of 10^{-6} M. To follow the reaction of these high enzyme levels we used an Aminco-Morrow stopped-flow apparatus. Physiological concentrations of DPN+, pyruvate and chicken heart lactate dehydrogenase were added together at time zero, and transferred into one of the reservoirs of the stopped-flow apparatus. The other reservoir contained a DPNH solution. At varying pre-incubation times the activity of the enzyme was measured. The results, shown in Figure 5, clearly indicate that the enzyme is almost completely inhibited following 20 minutes of pre-incubation. This observation indicates, that the ternary complex may be formed under these conditions, and thus that the complex may be of physiological importance.

In order to ascertain if the lactate dehydrogenase in heart tissue is actually partly present in the form of a ternary complex the following experiment was performed: 2 grams of fresh chicken hearts were minced in a blender with 10 ml of phosphate buffer for 30 seconds. The suspension was then filtered over a Buchner funnel and the relatively clear extract was assayed in an Aminco-Morrow apparatus, which had been previously supplied with a solution of DPNH and pyruvate. Using this technique it was possible to measure the enzymatic activity within 3 minutes after the initiation of the tissue grinding. Figure 6 shows that the activity increased significantly upon standing. Maximum activity was achieved following a dialysis overnight, and was found to be almost 300% of the original activity. This indicates that at least 65% of the lactate dehydrogenase in the heart muscle is present in an inactivated form.

2. Investigation of Mitochondrial Energy-linked Pyridine Nucleotide Transhydrogenation: The initial rate of the mitochondrial energy-linked pyridine nucleotide transhydrogenase reaction (equation 1) has been studied by stop-flow spectroscopy. This was accomplished by utilizine a TPN+ analog, thio-TPN+, as a substrate. Thio-TPN+ has a reduced absorption spectrum sufficiently different from DPNH so that its reduction can be measured independently from the oxidation of DPNH.

(1) $DPNH + TPN^+ + X \sim Y \quad DPN^+ + TPNH + X + Y$

In equation 1, $X \sim Y$ represents a non-phosphorylated high-energy intermediate associated with the process of oxidative phosphorylation which is formed as a result of succinate (or other substrate) oxidation or ATP hydrolysis.

A lag (20-50 sec) in thio-TPN^+ reduction was observed when the reaction was initiated by the addition of either succinate of ATP. However, no lag was observed if the mitochondrial membranes were preincubated with succinate or ATP, indicating that the formation of X Y, and not the formation of modified pyridine nucleotides, is initially rate limiting. Conformational changes in the membrane resulting from energization by succinate or ATP, measured with the fluorescent probe, 8-anilino-1-napthalene sulfonic acid (ANS), showed no lag and were half-maximal after 5 sec. In 98% D_2O, the transhydrogenase lag time was doubled, but the rate of conformational change was relatively unaffected. These and other studies indicate that the high-energy intermediate reactive in transhydrogenation is different from and in equilibrium with the energized intermediate(s) of oxidative phosphorylation.

3. Investigation of Mitochondrial Malate Dehydrogenase: Mitochondrial malate dehydrogenase, like heart type lactate dehydrogenase, is an important enzyme to be utilized for the clinical diagnosis and management of acute and healing myocardial infarction, or of pre-infection angina. Although it has never been shown, on one might expect to find that this enzyme has particular value in that it is mitochondrial bound, and therefore it would only be released from severaly ischemic tissue, which is a prognostically useful finding.

The pyridine nucleotide coenzyme-linked dehydrogenase, malata dehydrogenase, has the property of being inhibited by high concentrations of oxalacetate and it has mitochondrial and supernatant isozymes. The substrate inhibition is related to the formation of an abortive ternary complex composed of mitochondrial malate dehydrogenase, oxidized diphosphopyridine nucleotide and oxalacetate. The compound has an absorption maximum at a wavelength near that of the reduced coenzyme. In addition, there is a binary compound formed between the oxidized coenzyme and oxalacetate, which has maximum absorption at the wavelength of the maximum

absorption of the reduced coenzyme. The kinetics of the formation and of the breakdown of the binary and the ternary compounds have been carried out using stopped-flow techniques for the reactions involving both DPN and 3-Acetylpyridine DPN compounds.

The results show that the formation of bhe binary compound is base catalyzed. The formation of the ternary compound appears to proceed in two steps, one of which is rapid followed by a slow step, and the fast step might be related to the formation of the binary compound. The compound formed by the addition of the enzyr to the binary compound is identical to the ternary complex. Detailed studies are now underway to elucidate the intermediate steps in the formation of these compounds.

An extensive series of testing, redesigning and retesting has been carried out on a small simple stopped-flow pH-thermal apparatus. Two types of thermal detectors have been tested. The first was a thermistor unit which demonstrated that sensitivities of 100 microcalories could readily be achieved in the rather slow time response of the system, i.e., 50 milliseconds. Furthermore, problems developed with acid and alkali attacking the thermistor thru its glass envelope so that over several weeks of constant use troubles of reduction in sensitivity and increase in noise developec A new commercial unit was secured and is presently under going tests. In addition, bare thermistors have been obtained from two manufacturers and hopefully will be made into probes since it is highly desirable to get the 10% to 90% response time in 25 milliseconds. Present work has been carried out in a 3 mm diameter tube. Even so, only 100 microliters of each reagent was required. By making the probes much smaller, the internal diameter of the observation tube can be made only 1.5 mm in diameter, which should give a considerable improvement.

The second unit tested was the thin-film temperature sensor developed for us by the Jet Propulsion Lab. The speed of response of the Paralyene C coated units is 100 microseconds with a sensitivity of 150 mv/^{0}C out of the temporary bridge-amplifier they built for us. Because of the high second Harmonic content of the bridge, it was impossible to accurately determine the noise of the unit. We can say that it is less than 100 microvolts, but how much will have to await a better bridge. A new mounting was made so that it could be

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used in the high-speed stopped-flow thermal apparatus and this will be tested in the next year. At the same time a new stronger thermocouple has been obtained for this unit and will also undergo durability tests.

The stopped-flow pH apparatus has been extensively tested with the following results: Small flow system, 2 mm glass electrodes, Ingold Type lot #203-GA 31 or 32 referenced against a similar electorde gave reasonable results with inorganic reactions but became very sluggish after 4 or 5 shots with proteins. Cleaning with Clorox restored both response and sensitivity. However, it was found that unless the reaction vessel is completely isolated from ground, it is impossible to maintain a known reference level. Furthermore, the reference glass electrode must be flushed after each reaction. This was also found in the large apparatus with the 8 mm Ingold Electrode Type lot #202-LC, although the fast flow velocity and large area apparently produces much better cleaning action as it was only necessary to clean with Clorox at the end of the days work. While both the thermal and pH system work, considerable refinement will be needed to make them working instrument.

The purpose for which the high-speed flow system was originally designed was the measurement of the "on" constant for oxyhemoglobin. These measurements were made and published several years ago before the importance of 2-3 DGP was recognized. During the past year, we have been engaged in the preparation of truly 2-3 DPG isoionic hemoglobin. An excellent preparation has now been made and an extremely sensitive test for 2-3 DPG worked out. At the present time we have been able to demonstrate that at least 2 moles of 2-3 DPG are bound to reduced hemoglobin and that one of these molecules is very tightly bound and can only be removed enzymatically with a mutase of by dialysis in 3 M NaCl.

Using this preparation the "on" kinetics of Hb + CO and Hb + O_2 have been determined with and without 2-3 DPG with isoionic hemoglobin and no buffer. The results, in agreement with Antonini and Brunori (1) on CO, show that there is a three-fold increase in the "on" rate. We find this to also be true for O_2. Kilmartin and Rossi-Bernardi (2,3) have demonstrated that CO_2 reacts with the terminal α-amino groups of both the α and β chain but that the affinities are different. Riggs (4) has shown that 2-3 DPG, ATP and Phosphate compete for the CO_2 site. It is not clear if this is

the β chain α-amino or the α chain or both. Doubly blocked and singly blocked hemoglobin is presently being prepared by Kilmartin and Rossi-Bernardi to determine this. In order to clarify the dissociation curve we plan to run kinetic tests on our hemoglobin with and without 203 DPG, ATP and in the presence and absence of CO_2-Bicarbonate. These experiments will be reported on single and doubly blocked hemoglobin supplied by the Kilmartin-Rossi-Bernardi group. The importance of a clear understanding of this problem is extremely important for the understanding of a number of clinic management problems in respiratory physiology.

A second question which has arisen and is now being pursued is the question of reported increased glycosis in sickle cells, which other workers claim gives an increased 203 DPG level (5,6). We will repeat these experiments, while at the same time explore the possibility of using appropriate inhibitors to reduce 2-3 DPG levels in the cell thus reducing the tendency to sickle. This may offer some hope for the clinical management of Sickle Cell Anemia. This will be done in cooperation with E. Antonini (Rome).

References:

(1) Antonini, E. and Brunori, M. "On the Rate of Reaction of an Organic Phosphate (ATP) with Deoxyhemoglobin" FEBS Letters, Vol. 7, 352-352, 1970.

(2) Kilmartin, J.V. and Rossi-Bernardi, L. In Carbon Dioxide: Chemical, Biochemical and Physiological Aspects, P. 73 (NASA No SP-188), U.S. Government Printing Office, Washingto D.C.

(3) Kilmartin, J.V. and Rossi-Bernardi, L. "The Binding of Carbo Dioxide by Horse Hemoglobin" Biochemical Journal, In Press, 1971.

(4) Tomita, S. and Riggs, A. "Studies of the INteraction of 2-3 DPG and CO_2 with Hemoglobin from Mouse, Man and Elephant", JBC 246, 547-559, 1971.

(5) Charache, S., Grisolia, F., Fiedler, A.J. and Hellegers, A.E "Effect if 2-3 DPG on Oxygen Affinity of Blood in Sickle Cel Anemic", J. of Clinical Invest., 49, 806-812, 1970.

(6) Bunn, H.F. and Briehl, R.W. "The Interaction of 2-3 DPG with Various Hemoglobins", J. of Clinical Invest., 49, 1088-1095, 1970.

Current Research:

1. Stopped flow measurements using the polarization system are being conducted to determine detection levels and general usefulness.

2. Thermistor probes are being built for the two stopped-flow machines (they can be interchanged) and they will be tested for stability, reproducivility and sensitivity as well as speed of response. Using large scale-high purity enzymes prepared by the biochemistry group, a series of tests will be performed to determine the instruments applicability to clinical chemistry problems where rapid (1 per second), small sample (25 micro-liters) and high sensitivity (2% or normal serum levels at a 100 to 1 dilution) are needed.

3. A Calomel reference system and a completely isolated reaction cell will be tested in the hopes of eliminating the physical problems in stopped-flow pH. Several turpid solution reactions will then be used to test the system for direct cellular studies.

4. The application of the various flow systems to problems of both biochemical and clinical interests will continue with particular emphasis on the correlation in the rise of heart muscle LDH during myocardial infarction and the extent of cell rupture and resultant organ damage.

Honors: Invited Lecturer to the Universities of Cambridge, Milan and Rome.

Publications and Papers:

1. J. Everse, R.L. Berger and N.O. Kaplan, "Physiological Concentrations of Lactate Dehydrogenase and Substrate Inhibition", Science, 168, 1236 (1970).

2. J. Everse, R.L. Berger and N.O. Kaplan, "Complexes of Pyridine Nucleotides and their Function", in a symposium on Respiratory Enzymes (In honor of H. Theorell), In Press .

3. J. Everse and R.L. Berger, "Function of Complexes of Pyridine Nucleotides", Invited paper, American Chemical Society Meeting, September, 1970, Chicago.

Serial No. NHLI-273
Laboratory of Technical Development
Bethesda, Maryland 20014

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Development of Stopped-Flow Micro-Calorimetry for the Study of Biochemical and Cellular Reactions

Previous Serial No.: NHLI-192

Principal Investigator: Robert L. Berger

Other Investigators: W.E. Evans, M. Marini, N. Davids

Cooperating Units: Southern Regional R search Laboratory, Department of Agriculture, New Orleans, Louisiana; S. Hobbs, Research Instrument Shop, School of Medicine, Univeristy of California at San Diego; Department of Biochemistry, School of Medicine, Northwestern University; Department of Eng. Mech., Penn State University.

Project Description:

Objectives: Virtually all chemical reactions produce heat and calorimetry has long been used to investigate them. For biological use, however, high sensitivity, small volumes of reactants, and short equilibration times are needed. It is the objective of this project to develop such an instrument for use in the time range of a few seconds to 1 or 2 hours.

Methods Employed: Initial designs are constructed in this laboratory with special assistance from commercial firms in the construction of sensors; contracts are let, where warranted, for the development of a completed instrument with refinements that would tax our own facilities. The instrument is then tested in conjunction with other interested biochemical calorimetists utilizing appropriate enzymatic and cellular reactions.

Major Findings: The attempt to obtain thermopiles commercially for the Calvet-Evans Calorimeter has been abandoned and their construction has been carried out in the Research Instrument Shop of the School of Medicine, UCSD. Excellent units have been made and the detailed procedure documented. Final assembly is currently underway.

The differential mixing calorimeter has been tested as to its applicability for use on reactions of interest clinically.

Preliminary tests indicate that glucose can be determined on a 0.01 milliliter sample to a ± 2% using glucose oxidase and catalase. An investigation of the various factors which might produce interference is being carried out. The very slow equilibrium time (1-1/2 hours of this unit make it very difficult to do more than 2 to 3 experiments per day. This was one of the major reasons for the construction of the Calvet=Evans unit with a stopped-flow ball mixer sampling unit. None the less, it is clear from these initial experiments that the thermal method offers a great practical advantage in that greatly simplified chemistries may be performed.

Work has progressed on the thermal titration apparatus to the point that a full scale sensitivity of 4 millicalories with an electrical noise of about 30 microcalories peak-peak has been achieved. The thermostating system produced much larger variations, i.e, about 1 millicalorie per 5 minutes. An improved thermostat has been installed. The thermistor used was the same one as used for the stopped-flow system. Tests are underway with the new unit and a full field test on an enzyme system will be conducted.

Current Research:

A working unit will be ready by June 1st. Testing will be carried out on standard reactions. The utilization of this instrument to study the feasibility of using thermal methods for many of the tests now used in clinical medicine will be undertaken. Preliminary work has indicated the method is excellent for glucose determinations using glucose oxidase.

Honors and Awards: None

Publications:

1. S.K. Devi, R.Moore, N. Davids, A.F. Findeis, and R.L. Berger, "Simulation of Water Movement Across the Cell Membrane in a Hypertonic Medium" Comput. Biol. and Med. Vol 1, p. 141-155, 1970.

Molecular Disease

ANNUAL REPORT OF THE
MOLECULAR DISEASE BRANCH
NATIONAL HEART AND LUNG INSTITUTE

July 1, 1970 through June 30, 1971

The Molecular Disease Branch contains six sections; two of these are autonomous: the Section on Lipid Metabolism, and the Section on Human Genetics. The others are coordinated into a working group primarily concerned with plasma lipoproteins and genetically determined disorders of lipid metabolism. The latter group also maintains an inpatient and outpatient clinical service which also permits clinical investigation of hematologic problems by staff of the Section on Human Genetics. A coordinate program with the Section on Peptide Chemistry, EB, is maintained whereby some aspects of the structure of plasma lipoproteins are further explored, particularly the determination of covalent structure of the apolipoproteins.

Activities Related to Plasma Lipoproteins and Molecular Diseases
(Sections headed by Drs. Fredrickson, Levy, Gotto and Sloan)

Apolipoproteins. Much of the present work is concentrated upon the apoproteins in lipoproteins of which no less than nine are isolable. Four of these were first isolated in this laboratory. These (identified by their carboxyl-terminal residues and followed by designation of the lipoprotein families in which they appear) are: Apo-val, -glu, -ala_1, and -ala_2 (VLDL and HDL), -ser(LDL), and -gln, -thr_1, -thr_2, -thr_3 (HDL). The amino acid sequence of Apo-ala_2 will be completed in several more months; it differs from Apo-ala_1 only in sialic acid content. It has been shown that Apo-ala probably contains a single glycopeptide; that Apo-val contains no carbohydrate; the sugar content of -glu is low, but still uncertain. These three major VLDL apoproteins bind lipid avidly. Last year, this and another laboratory discovered independently that Apo-glu is a potent activator of the important enzyme lipoprotein lipase. The availability of pure apoproteins and specific antisera to them has permitted continued exploration into their functions. This includes assistance in establishing that at least two forms of triglyceride lipase activity are present in tissues and transiently appear in plasma after heparin injection. It has also been shown that normal amounts of the VLD apoproteins are present in plasma of patients with type I hyperlipoproteinemia and with abetalipoproteinemia. Their presence in the first dispels possible absence of lipase activator as cause of this disease. Their presence in abetalipoproteinmia has led us to conclude that in this mutant, Apo-ser formation or utilization is primarily affected and, moreover, that Apo-ser is the apoprotein most critical for glyceride transport from cells. Pure apoproteins from all of the lipoprotein

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families have now also been prepared from rat plasma compared with human apoproteins. They are also being used in studies of either whole animals or perfused intestines or livers to determine which apoproteins are common to VLDL and chylomicrons, their primary source and fate during metabolism.

Much has also been learned about HDL in the past two years. Most of its apoprotein content is due to Apo-thr and Apo-glu. The latter has been degraded by pronase to four fractions and the affinity for lipid of these and of the parent apoproteins extensively studied. Several aspects are of particular interest: The ability of apolipoproteins to compete with an enzyme (α-hydroxybutyrate dehydrogenase) for lipid forms a suitable assay for relative lipid affinity; the ability of HDL to inactivate endotoxin allows test of complementarity of apoproteins; and studies of the reformation of lipids and apoproteins into lipoproteins in vitro reveal much of the requirements for stability and properties of the native complexes. Circular dichroic spectra and appearance under the electron microscope suggest that apoprotein, phosphatidyl choline and cholesteryl esters are required for complete reorganization of the secondary and tertiary structure of recombined HDL. Selective affinity chromatography of HDL has been used to prepare separately Apo-thr- and Apo-gln-subunits to determine whether both apoprotein occur in all HDL complexes or these lipoproteins circulate as mixtures of complexes differing in apoprotein content. Electron spin resonance has been used to show that recombined lipoproteins are not identical to native lipoproteins.

New Lipoprotein: A new phenomenon, "sinking pre-beta lipoprotein" was clarified this year. This is triglyceride-poor lipoprotein having pre-beta mobility and a density of between about 1.05 and 1.08 gm/ml. The incidence of this phenomenon was found in about 10 percent of over 3,000 plasma samples, shown to distribute as a mendelian dominant trait, and indiscriminately among normal subjects and patients with different types of hyperlipoproteinemia. This is the explanation for some "double pre-beta bands" described by many using agarose electrophoresis of lipoproteins; it is also an additional reason for using triglyceride concentrations to interpret the significance of prominent pre-betalipoprotein bands, one of the laboratory's dicta for "phenotyping." It was then shown by preparing a specific antiserum that sinking pre-beta is identical to the Lp(a) antigen described a number of years ago by Berg as an example of beta-lipoprotein polymorphism. The interpretation of Lp(a) as being present only in bearers of a particular allelic gene has been questioned by others. We also found, with highly sensitive assays employing counter-immunoelectrophoresis, that Lp(a) is detectable in at least 80% of subjects. Pure Lp(a) apoprotein

has been prepared by affinity chromatography. As a possible "proto-β-lipoprotein" or fragment of β-lipoprotein, it is an exciting object of continuing study in this and other laboratories.

Lipoprotein Turnover and Metabolism. Human VLDL has been successfully tagged with ^{125}I and re-injected in subjects. The label is distributed among a number of the apoproteins and the appearance and decay of radioactivity in each and in other lipoproteins has been followed for many days. These experiments have been of particular interest in reference to type III hyperlipoproteinemia. Our previous studies having failed to detect an abnormal apoprotein in the circulating "floating beta lipoprotein" that is characteristic of this disease, attention has turned to a possible defect in the normal conversion of VLDL to LDL. "Floating beta" lacks the typical VLDL apoproteins, contains only Apo-ser, but has more glyceride than normal LDL. The sequential course of radioactivity in plasma of controls indicates that conversion of VLDL does indeed progress to LDL through an intermediate of Sf > 12. This intermediate stage is greatly prolonged in type III. The metabolic defect responsible now becomes the object of intense exploration.

Familial Hypolipoproteinemia. The laboratory continues to screen and study such patients and their families with a particular emphasis on sharpening phenotyping and scrutinizing our system of classification. The latter was "adopted" as a reference by WHO last summer and, in this regard, we have "officially" broken out two subtypes of type II (IIa, LDL increased alone, and IIb, with additional increase in VLDL.) Three major projects begun this year are of particular note: (1) pursuit of complete information about vascular involvement of 120 kindreds affected with type II, a study now over half completed; (2) intensive planning and organization of an intervention study in familial type II (in partnership with the Cardiology Branch), to use coronary angiography as an end point in a double-blind comparison of cholestyramine and placebo in patients with prior evidence of coronary artery disease; and (3) assessment of the need and interest with the American scientific community for resources for lipid and lipoprotein measurements and facilities for managing hyperlipoproteinemia - as a necessary step toward prophylaxis against premature vascular disease - and management by a staff member (Dr. Levy) of a program to establish a network of such "Lipid Clinics" within the Extramural Programs of the Institute.

Liver Cell Culture. Embryonic mouse liver cells (the Weymouth line) have been started and propagated in tissue culture as a potential model for study of many aspects of lipid and lipoprotein metabolism. The propagated cells (no longer diploid) have already been shown to synthesize briskly cholesterol, cholesteryl esters, triglycerides and various phospholipids.

Further Studies of Molecular Diseases. Much effort has been made to cultivate many lines of fibroblasts mutant for different lipid storage disease. One group of particular interest are cells from each of the four types of the sphingomyelin lipidoses which have been used to establish which types are, and which are not, deficient in sphingomyelinase activity. We now have the largest collection of type B (deficient) and types C and D (not deficient) cultures. Tests for all of the enzymatic defects in the lipid storage diseases are now available for prenatal counseling. Of greatest interest is the successful near-purification of both normal and mutant enzymes from patients with two of these diseases. Affinity chromatography has been used to isolate both sphingomyelinase and cerebroside sulfatase (aryl-sulfatase A). The aim is a search for the different structural alterations in these enzymes directed by mutation and responsible for catastrophic decrease in activity.

Annual Report of the
Section on Metabolism
Molecular Disease Branch
National Heart and Lung Institute
July 1, 1970 through June 30, 1971

General

The Section on Metabolism is concerned, as previously, with the mechanisms underlying hormonal control of lipolysis in fat cells. The role of cyclic AMP and cyclic GMP in this and other tissues and processes is of major interest, with attention focused in the past year on cells in culture and phagocytic cells (as well as fat cells). Investigation of a number of other aspects of the phagocytic process in granulocytes and alveolar macrophages was made possible by the development of new methods for the isolation of phagocytic vesicles and for assay of the rate of phagocytosis. Studies on dipalmitoyl lecithin, the active component of pulmonary surfactant, and on the regulation of cholesterol synthesis in mammalian cells have been continued.

A. Mechanism of hormonal regulation of lipolysis in fat cells

We have found that hormone sensitive lipase in the soluble fraction of adipose tissue homogenates can be inactivated by incubation with ATP and $MgCl_2$. In order to demonstrate inactivation, after partial purification of the lipase by ammonium sulfate precipitation, it was necessary to add another fraction, the active component of which is lost with repeated freezing and thawing, heating or dialysis. Using these fractions, the effects of pH, nucleotides, ions, and the concentration of the several components of the system on the rate and extent of lipase inactivation have been studied. After inactivation with ATP and $MgCl_2$, partial reactivation of the lipase has been achieved by incubation with ATP, $MgCl_2$, cyclic AMP and a partially purified cyclic AMP-dependent protein kinase from skeletal muscle.

After investigation of the effects of glucagon on lipolysis and on adenyl cyclase activity in fat cells, we have concluded that there is a selective loss of fat cell glucagon receptors with growth in the rat. No similar age-related change in the response of heart adenyl cyclase to glucagon was demonstrable.

It has been known for several years that insulin inhibits lipolysis in fat cells, perhaps through its demonstrated effect on intracellular cyclic AMP concentration. Whether the latter effect results from an insulin-induced increase in cyclic AMP degradation or inhibition of its formation is unknown. Since completion of studies on the action of several so-called lipolytic hormones that increase cyclic AMP levels by stimulating adenyl cyclase activity, a systematic investigation of the effects of insulin on protein kinase, adenyl cyclase and phosphodiesterase activity has been initiated.

B. Formation, degradation and action of cyclic AMP and cyclic GMP

This year, after comparing the two procedures for a time, we discontinued using the phosphorylase bioassay for cyclic AMP (Sutherland) and are now using only the protein binding method of Gilman (NHLI). We have devised a simple procedure for purifying cyclic GMP from tissues and a specific protein binding assay for this nucleotide, and are now able to investigate the effects of hormones and other agents on cyclic GMP levels in fat cells and lung, as well as in mammalian cells in culture.

1. Cells in culture: Mouse fibroblasts (L-cells) respond to prostaglandin E_1 (PGE_1) with a 10-20 fold increase in cyclic AMP concentration, whereas epinephrine produces only a small elevation. Contrary to earlier reports, we have found that in hepatoma cells that respond to corticosteroids with an increase in tyrosine aminotransferase, epinephrine (but not glucagon or PGE_1) causes a 2-3 fold elevation in cyclic AMP content. The presence of theophylline, an inhibitor of cyclic nucleotide phosphodiesterase, was required for this effect, whereas in L-cells, theophylline enhanced only slightly the response to epinephrine or PGE_1. The effect of epinephrine was prevented by propranolol, but not by insulin.

2. Leukocytes: It has been reported that during phagocytosis of polystyrene beads by leukocytes, there is a marked increase in cyclic AMP concentration. These studies were carried out with leukocytes from human peripheral blood. Such preparations contain several types of cells including platelets. We have separated human polymorphonuclear leukocytes (PMN) (85-95% pure) and find that in these cells cyclic AMP levels rise only slightly during phagocytosis, while in a mixture of all other cell types present in the leukocyte preparation before fractionation, there is a large increase. In relatively pure populations of rabbit alveolar macrophages and guinea pig polymorphonuclear leukocytes (peritoneal exudates) cyclic AMP levels did not significantly increase during phagocytosis. It appears that phagocytosis probably does not lead to elevation of cellular cyclic AMP concentrations even in human PMN. These findings, as well as those with mast cells (v.i.) emphasize again the difficulties (and pitfalls) in attempting to relate physiological processes to changes in cyclic AMP content in mixed populations of cells.

3. Mast cells: On the basis of earlier studies here and elsewhere, a role for cyclic AMP and catecholamines in modulation of histamine release has been postulated. Dextran induced release of histamine from rat peritoneal mast cells, was inhibited by dibutyryl cyclic AMP, theophylline, 10^{-8} M isoproterenol and by epinephrine and norepinephrine at higher concentrations. When incubated with isoproterenol plus theophylline, there was a considerable increase in cyclic AMP content of the cell suspension. The preparations of cells used in these studies contained, however, large numbers of non-mast cells, and only minimal changes in cyclic AMP levels were found in purified preparations of mast cells (which also did not liberate histamine in response to dextran).

C. Metabolism of lung and phagocytic cells

1. Isolation of phagocytic vesicles: A method has been devised for the isolation of intact phagocytic vesicles (PV) in a high state of purity from cells that had ingested droplets of paraffin oil emulsified with serum albumin. The paraffin oil was colored with Oil Red O for quantification of phagocytosis. PV isolated from alveolar macrophages and from human and guinea pig leukocytes were characterized and compared in terms of content of paraffin oil, protein, cholesterol, phospholipids (total and individual) and several enzymes. PV from guinea pig PMN contained acid and alkaline phosphatase, β-glucuronidase, peroxidase and NADH oxidase activities. With phagocytosis there was an equivalent loss of these enzymes from the granule fraction with no change in total cell activity or transfer of enzymes into the soluble fraction. PV from human leukocytes were very similar in composition to those from guinea pig cells.

Alveolar macrophages did not contain measurable alkaline phosphatase or peroxidase activities. In contrast to the PMN catalase which was entirely soluble, about half of the macrophage catalase was sedimentable in homogenates of resting cells. A portion of this was transferred to the PV with phagocytosis along with acid hydrolases from the pellet fraction as in the leukocytes. Alveolar macrophages from rabbits treated with BCG had greater activity of acid hydrolases than did cells from normal rabbits and incorporated considerably more of these enzymes into PV.

2. Degranulation during phagocytosis: Determination of the enzyme content of PV isolated from guinea pig PMN at several times during phagocytosis revealed that degranulation was closely coupled temporally to particle uptake (formation of PV) and when phagocytosis was terminated, little further degranulation occurred. The rates of transfer of β-glucuronidase, peroxidase and NADH oxidase were similar to each other and different from those of acid and alkaline phosphatases, probably a reflection of the enzymatic heterogeneity of the lysosome-like granules in those cells. It has been suggested that PMN from patients with chronic granulomatous disease do not degranulate normally, but enzyme activities (per unit of protein or Oil Red O) in PV from cells of three patients with this disorder were not different from those in PV from normals; thus no defect in degranulation was evident. The specific activities of β-glucuronidase and peroxidase in PV from two patients with Chediak-Higashi syndrome were, however, low.

3. Oxidases in phagocytic cells: It has been suggested that cyanide-resistant pyridine nucleotide oxidases play a part in the generation of hydrogen peroxide during phagocytosis. Two fluorometric assays were developed for assay of these enzymes. Homogenates of PMN (and fractions thereof) displayed considerably more activity toward NADH than toward NADPH. Study of the NADH oxidase activity was complicated by marked instability of the enzyme(s) and anomalous activity vs. substrate behavior. In whole homogenates, there were apparently two Km's for NADH, but only the lower Km activity was found in the phagocytic vesicles, which contained no detectable NADPH oxidase activity. In subcellular fractions of leukocytes from patients with chronic granulomatous disease, which failed to produce hydrogen peroxide during phagocytosis, NADH oxidase activity was not different from normal.

PMN incorporate iodide into protein during phagocytosis, presumably by generating hydrogen peroxide in the presence of peroxidase. After phagocytosis in the presence of I^{125}-iodide, iodinated protein was found only in the phagocytic vesicle fraction, which is consistent with the hypothesis that iodination is an antimicrobial concomitant of phagocytosis.

4. Rate of phagocytosis: A simple, sensitive assay for rate of phagocytosis was developed using as substrate paraffin oil colored with Oil Red O emulsified with albumin. Optimal conditions (medium, time, etc.) for phagocytosis by guinea pig PMN were established. Stimulation of glucose oxidation was directly proportional to the initial rate of phagocytosis. Colchicine, N-ethyl maleimide, theophylline, dibutyryl cyclic AMP and hydrocortisone all decreased the phagocytic rate. These agents, in the concentrations employed, had previously been thought to produce metabolic effects without inhibiting phagocytosis. Insulin slightly but consistently enhanced the phagocytic rate. Phagocytosis of the standard emulsion prepared with albumin was markedly dependent on the presence in the medium of Mg^{++}. Results of studies of the rate of uptake of emulsions prepared with a variety of other stabilizers (albumin derivatives, other proteins, starch) with and without Mg^{++}, are consistent with the view that the acceptability of a particle for ingestion depends on a complex interaction between the surface of the particle and divalent cations in the medium to produce a favorable net surface charge.

5. Opsonization and phagocytosis: Guinea pig PMN ingested paraffin oil emulsified with E. coli endotoxin only after opsonization with serum. Modification of the serum, e.g., heat or zymosan treatment, altered the rate of phagocytosis of the emulsion in the same manner as these conditions have been shown to modify the uptake of E. coli organisms. This may be a useful model system for investigation of immunological events related to phagocytosis.

6. Dipalmitoyl lecithin in lung and phagocytic cells: Studies on the synthesis of dipalmitoyl lecithin (DPL), the active component of pulmonary surfactant, by lung and alveolar macrophages, were completed this year. Pulmonary lavage fluid from steroid-treated rabbits contained more protein, phospholipid and macrophages than did the washings from control rabbits, but no selective effect of corticosteroid (or of thyroxine) on the content or synthesis of DPL in whole lung was demonstrable.

Although the amount of DPL per mg of cell protein is considerably lower in rabbit heterophils than it is in alveolar macrophages, the percentage of total lecithin that is dipalmitoyl is similar in the two types of cells. In both types of cells, the lecithin of the phagocytic vesicle fraction relative to whole cells is enriched in DPL. We have found a high degree of saturation of the phospholipids of phagocytic vesicles, contributed to in part by DPL, which would tend to make the vesicle membrane relatively immune to peroxidative damage. The latter is presumably related to the augmentation of oxygen consumption that is associated with phagocytosis. Perhaps rather than serving as a source of pulmonary surfactant, the DPL of the alveolar macrophage is related to the phagocytic function of this cell.

7. Effects of oxidant gases on function of alveolar macrophages: Impairment of bacterial clearance and ultrastructural changes in alveolar macrophages occur in mice exposed for prolonged periods to 100% oxygen. Tolerance can be induced by prior intermittent exposure to 100% oxygen. Analyses of the pulmonary lavage fluid from mice used in these collaborative studies are being completed.

D. Regulation of cholesterol synthesis in mammalian cells

Some characteristics of feedback regulation of cholesterol synthesis in fibroblasts grown in culture were reported last year. This year the behavior of cells derived from skin biopsies of patients with Type II and Type III hyperlipidemia was compared with that of cells from normal humans, and no differences were noted. It was found that cholesterol synthesis by leukocytes from peripheral blood of normal humans is likewise inhibited by exogenous sterol, e.g., by incubation with whole serum. (Delipidated serum stimulates synthesis in both types of cells.) In contrast, lipid synthesis in rabbit aorta (mince or strips) was stimulated after incubation of the tissue with whole serum, albeit to a lesser extent than with delipidated serum. It appears that feedback inhibition of cholesterol synthesis may be of less importance in aortic tissue than it is in fibroblasts and leukocytes.

Annual Report of the
Section on Human Biochemistry
Molecular Disease Branch
National Heart and Lung Institute
July 1, 1970 through June 30, 1971

The mechanism and regulation of mammalian protein synthesis has been investigated by utilizing hemoglobin biosynthesis as a model system. The approach has been to develop active cell-free protein synthesizing systems from rabbit reticulocytes and from human reticulocytes. By fractionating these cell-free systems it has been possible to identify several of the cellular components involved in the process of initiation of hemoglobin synthesis and to identify the molecular defect in the human genetic disease β-thalassemia.

A summary of our results obtained over the past twelve month follows:

1. Three protein initiation factors, M_1, M_2, and M_3, have been partially purified and characterized. M_1 is a binding factor M_2 is a factor which specifically recognizes the mammalian initiator tRNA; and M_3 is a factor which is specific for natural mRNA templates. The factor M_2 has been separated into two components, M_{2A} and M_{2B}, which differ in their enzymatic properties. Similar initiation factors are being isolated from rabbit liver cells. The properties of the liver factors are being compared with those of the reticulocyte factors.

2. The initiator tRNA for hemoglobin biosynthesis has been identified as Met-$tRNA_F$. Not only is Met-$tRNA_F$ specifically bound to reticulocyte ribosomes by the initiation factors M_1 and M_2, but also Met-$tRNA_F$, under the direction of the initiation factors, donates the first amino acid of the nascent hemoglobin chain.

3. A highly active human cell-free system has been developed which is able to reproduce the globin synthesis properties of intact cells. By utilizing the various components from normal human and thalassemic human reticulocytes, it has been shown that the molecular defect in β-thalassemia resides in the thalassemic β-chain mRNA. By utilizing the mRNA purified from thalassemic reticulocytes in a system containing otherwise completely normal human cell components, it is possible to reproduce the α/β chain abnormality seen in the intact thalassemic reticulocyte.

Serial No. NHLI-274(c)
1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: The contribution of carbohydrates to the structure and function of the plasma lipoproteins.
Previous Serial Number:	NHLI-300(c)
Principal Investigators:	Howard R. Sloan, M. D., Ph. D. Donald S. Fredrickson, M. D. Robert I. Levy, M. D.
Other Investigators:	Seniye Temel, B.S. Barbara Davis, B.S. Samuel Lux, M.D. Peter Herbert, M.D. Stephen Demosky, B.S.
Cooperating Units:	None

Project Description:

Objectives: The primary aim of this study has been an investigation of the carbohydrate composition of the human plasma lipoproteins. It is hoped that by so doing, some insight can be gained as to the role played by the carbohydrate portion of the plasma lipoproteins. This information may provide greater understanding of the mechanism of normal lipid and glycolipid transport.

Methods: Preparation of lipoproteins and their apoproteins. Plasma was collected from normal subjects and from patients with Type IV and V hyperlipoproteinemia. The lipoprotein families, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) were prepared by ultracentrifugal flotation at $d < 1.006$ gm/ml, $1.019 < d < 1.063$ and $1.063 < d < 1.21$, respectively. The apoproteins or protein moieties of the lipoproteins were obtained by exhaustive delipidation with chloroform: methanol. The glycolipids were

isolated from the chloroform:methanol extracts by chromatography on columns of silicic acid and thin-layers of silica gel G. The unique VLDL apoproteins, ApoVLDL-val, glu and ala were isolated from VLDL employing methods described in previous annual reports. The two HDL peptides, R-thr and R-gln were isolated by chromatography on columns of Sephadex G-200 and DEAE-cellulose. Glycopeptides were prepared from LDL by extensive digestion with the proteolytic enzyme pronase. The glycopeptides were isolated and purified by chromatographic techniques.

Analytical Techniques. A large number of analytical techniques were employed to investigate the carbohydrate composition of the lipoproteins. These included gas-liquid, thin-layer, and paper chromatography; enzymatic assays; amino acid analysis; and chemical assays.

Major Findings:

1. Mono-, di-, tri-, and tetrahexosyl ceramides are present on VLDL, LDL, and HDL. The bulk of the glycolipids are apparently carried in the plasma on LDL. Significant amounts are, however, present in each lipoprotein class. Ganglioside G_{M3} is also present in LDL.

2. Results of carbohydrate analyses on acid-hydrolyzed VLDL peptides are in annual report No.

3. R-thr isolated from HDL contains 0.4% (by weight) glucosamine, 0.7% mannose, and 0.4% glucose; there is no sialic acid.

4. R-gln isolated from HDL contains 0.5% (by weight) glucosamine, 0.9% galactosamine, 0.1% mannose and 0.4% glucose; there is no sialic acid.

5. The carbohydrate moiety of apoLDL consists of 1.0% (by weight) glucosamine, 0.6% N-acetylneuraminic acid, 1.9% mannose, and 1.1% galactose.

6. The glycopeptide isolated from LDL contains the same carbohydrates found in apoLDL. The ratios between the component carbohydrates of apoLDL and the glycopeptide isolated from apoLDL were quite similar. Aspartic acid (40%), serine (34%), glycine (12%) and threonine (10%) account for 96% of the amino acid content of the glycopeptide.

7. The carbohydrate moiety of apoHDL consists of 1.3% (by weight) glucosamine, 0.3% galactosamine, 0.4% N-acetyneuraminic acid, 0.7% mannose and 0.4% galactose.

8. The glycopeptide isolated from HDL contains the same carbohydrate found in HDL, aspartic acid, serine, threonine, alanine and glutamic acid account for 97% of the amino acid content of the glycopeptide.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the basic structure of the plasma lipoproteins is essential for our understanding of normal and abnormal lipid transport. This information is particularly pertinent to the study of vascular-disorders associated with abnormal lipid metabolism and transport.

Proposed Course:

1. Efforts will be directed toward the precise quantification of the amount of carbohydrate in each of the plasma lipoproteins and their constituent peptides.

2. The carbohydrate sequence of the LDL and HDL glycopeptide will be investigated.

3. The importance of the carbohydrate moieties with respect to antigenic reactivity will be evaluated.

4. The effect of changes in carbohydrate content on the antigenic reactivity and, when possible, the function of the plasma lipoproteins and apoproteins will be evaluated.

Publications:

Prescott, B., Chernick, S. S., James, W. D., Caldes, G., Barker, D., Sloan, H. R., and Chanock, R. M.: Mycoplasma Pneumoniae. Phosphatidyl Glycerol. Proc. Expt. Biol. Med. 134, 711, 1970.

Sloan, H. R., Kwiterovich, P. O., Levy, R. I., and Fredrickson, D. S.: Carbohydrate Components of Human Plasma Lipoproteins. Circulation, 42, 28, 1970. (Abstract).

Serial No. NHLI-275(c)

1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: The turnover and function of very low density lipoproteins
Previous Serial Number:	None
Principal Investigators:	Robert I. Levy, M.D. David W. Bilheimer, M.D. Shlomo Eisenberg, M.D.
Other Investigators:	Sheila Spivey, B.A. Betty Masket, B.S., M.A.
Cooperating Units:	None

Project Description:

Objectives: Very low density lipoproteins (VLDL) serve as the principal vehicle for transport of triglyceride from the liver (and gastrointestinal tract) to sites of metabolism and storage in other tissues. This study is part of a long term project designed to investigate the metabolic breakdown of VLDL and its relationship to other serum lipoproteins.

Present objectives are: (1) to investigate the behavior of ^{125}I-VLDL with relation to other lipoproteins in vitro; (2) to label isotopically and isolate pure fractions of VLDL apoproteins for study in VLDL metabolism in vitro and in vivo; (3) to investigate the metabolism of labeled VLDL and its apoproteins in animals; (4) to continue the investigation of VLDL turnover and its conversion to other lipoprotein subclasses in normal subjects and in patients with types I, III, IV and V hyperlipoproteinemia; (5) to establish the determinants of VLDL synthesis and catabolism in vivo.

Methods: VLDL was obtained from fasting normal subjects or patients afflicted with types I, III, IV or V hyperlipoproteinemia. The VLDL was isolated and concentrated by ultracentrifugation at salt density 1.006. Purity was established by immunochemical methods. Following iodination by the

iodine monochloride method, the material was sterilized by millipore filtration and tested for pyrogens and sterility before use. ^{125}I-VLDL for in vitro use was similarly prepared.

Iodinated VLDL for in vitro study by gel filtration and/or polyacrilamide gel electrophoresis was handled as previously described.

Major Findings:

1. Iodination of VLDL: The iodine monochloride method continues to be satisfactory in providing a radioactively labeled protein that is nonreutilizable and which behaves identically to the native VLDL when studied by immuno and paper electrophoresis.

A mean of 10% of the isotope attaches to lipid, and of those lipids labeled, phospholipid (lechithin) (50%), cholesterol ester (14%) and triglyceride (15-23%) are labeled most extensively.

2. Protein labeling: ^{125}I-apo VLDL has been extensively studied by polyacrilamide gel electrophoresis to establish the variation in isotopic labeling that occurs among the several peptides. The mean distribution is as follows: apoLDL 51%, apoHDL 4.5%, apoLP-ala + apoLP-glu 39%. Iodination has not altered the physical behavior of these VLDL apoproteins when examined by Sephadex gel chromatography and polyacrilamide gel electrophoresis.

3. Preliminary in vitro studies: When ^{125}I-VLDL is mixed with human plasma, there is transfer of radioactivity to other lipoproteins. Analysis of the other lipoprotein fractions in plasma indicated that about 3% attached to the Dl.006-1.019 lipoprotein fraction, 7% attached to LDL and 10-20% attached to HDL. One-fourth of the activity on LDL was due to transfer of lipid from VLDL, the remainder being due to apoLP-ala and apoLP-glu. About 75 - 80% of the radioactivity in HDL is found collectively in apoLP-ala + -glu. This transfer of peptides from VLDL to HDL is not due to lechithin-cholesterol acyltransferase (LCAT) because in the presence of inhibitors of this enzyme the transfer occurs to the same degree. These small peptides are capable of transfer from HDL to VLDL as well.

4. Human studies: ^{125}I-VLDL metabolism has been studied in 12 subjects. This group includes 3 normal volunteers, one patient with type I, 3 with type III, 4 with type IV and one with type V hyperlipoproteinemia. One type III patient received ^{125}I - VLDL from a normal subject. A type III and type IV patient participated in a cross over experiment in which each received his own as well as the other patient's VLDL in two successive studies. No adverse reactions have been noted in any study.

In all patients except the one with type I hyperlipoproteinemia, ^{125}I in VLDL rapidly declined. Radioactivity sequentially appeared in the intermediate lipoprotein fraction of density 1.006-1.019 (peak at 6 to 12 hours with rapid decline) and in LDL (D 1.019-1.063) (peak at 24 hours with slow decline). This metabolic sequence could be accelerated by intravenous heparin administration which was performed in both a type IV and a type V patient.

The type I subject was able to convert VLDL to LDL but at a much slower rate, such that the peak of radioactivity in the intermediate fraction occurred at day one and maximal activity occurred in LDL between the second and third day.

5. Analysis of the distribution of ^{125}I in the apoproteins of VLDL during in vivo metabolic studies revealed that the apoLDL portion of VLDL declined from 50% to 8% by 12 hours after injection while the activity in apoLP-ala + apoLP-glu increased from 20-30% at time 0 to >85% by 24 hours. The decay of apoLDL from VLDL occurred coincident with the rapid rise of activity in the intermediate and the slower rise in activity in plasma LDL.

These data provide strong evidence that LDL is derived from VLDL catabolism. In the type I patient, this conversion is delayed which confirms that triglyceride hydrolysis is involved in this metabolic process.

Significance to Biomedical Research and the Program of the Institute: Knowledge of the dynamics of VLDL metabolism as well as its determinants is fundamental to understanding of certain types of hyperlipoproteinemia that have an important relationship to atherosclerosis.

Proposed Course:

1. Studies of the turnover and interconversion of VLDL will continue. Efforts will be made to define the determinants of VLDL synthesis and catabolism.

2. Studies of VLDL metabolism in the several types of hyperlipoproteinemia mentioned above will continue. Special emphasis will be placed on type III which may be due to faulty conversion of VLDL to LDL with the resultant formation of an abnormal lipoprotein.

3. A computerized model of VLDL metabolism is being developed to help conceptualize and understand the processes involved in synthesis and clearance of this lipoprotein.

4. Studies will continue into the in vitro behavior of VLDL apoproproteins using tracer techniques. Reasons for transfer of peptides between VLDL and other lipoproteins will be sought.

5. Animal studies will be designed to look more closely at the organ systems involved in both VLDL synthesis and catabolism.

Publications:

None other than abstracts.

Serial No. NHLI-276(c)
1. Molecular Disease Branch
2. Section on Lipoproteins

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: The functional roles of plasma high density and low density lipoproteins
Previous Serial Number:	303 (C)
Principal Investigators:	Robert I. Levy, M.D. Donald S. Fredrickson, M.D. David W. Bilheimer, M.D. Shlomo Eisenberg, M.D.
Other Investigators:	Sheila Spivey, B.A. Betty Masket, B.S., M.A. Nancy Q. Mason, B.A. Margaret Hill
Cooperating Units:	Herbert Windmueller, Ph.D., Section on Nutritional Biochemistry, National Institute of Arthritis and Metabolic Diseases

Project Description:

Objectives: As part of a long term project concerned with both the function and metabolic fate of plasma lipoproteins, the turnover of beta lipoprotein (LDL) has been studied in normal and type II hyperlipoproteinemic subjects under a variety of conditions. It has been established that the elevated plasma LDL levels in type II hyperlipoproteinemia are due to impaired catabolism of this lipoprotein rather than to increased synthesis. It was further shown that cholestyramine lowers LDL levels in this disease by increasing the rate of catabolism. Nicotinic acid, in contrast, was found to impair synthesis while leaving catabolism unchanged and thereby caused lower LDL levels. This work has formed the basis for continued studies in LDL metabolism as well as expansion into studies of high density lipoprotein metabolism.

Objectives are: (1) to test the hypothesis that LDL is derived from VLDL catabolism by demonstrating that stimulation of VLDL production through carbohydrate feeding results in increased synthesis of LDL, (2) to investigate the relationship of bile acid formation to LDL metabolism,(3) to continue studies on the effects of diet and specific pharmacologic agents on synthetic and catabolic rates of the apoproteins, and (4) to begin studies of HDL metabolism in animals and man using radioiodinated HDL and its apoproteins.

Methods: The standard preparative techniques using plasmapheresis, preparative ultracentrifugation, paper electrophoresis, immunochemical identification and beta quantification have been previously described. The lipoproteins, following iodination and concentration, were iodinated by the iodine monochloride method. Following ultrafiltration, pyrogen testing, and bacteriologic screening, the radioiodinated lipoprotein was made ready for injection.

Radioiodinated HDL was delipidated and its apoproteins separated by Sephadex G-200 gel filtration. Additional separation of the mixture of apoLP-val + -glu + - ala was achieved by DEAE chromatography. The isolated peptides were incubated in plasma to determine their affinity for specific lipoproteins.

In animal studies (principally the rat), iodinated VLDL, LDL, and HDL were injected intravenously, and radioactive products followed for 72 hours. Animals were sacrificed at specified times and tissue analysis was performed to locate the principal sites of uptake of these lipoproteins. Rat ^{54}Cr-erythrocytes were also injected to permit correction for blood contamination in the organs assayed.

Major Findings:

1. In vitro iodination of lipoproteins by the iodine monochloride method continues to be satisfactory. No evidence of denaturation has been detected. When LDL is labeled, 98% or more of the isotope is attached to protein, and efficiency of labeling is 25-35%. Using HDL, 99% or more of the isotope is found on protein, and the efficiency is 60-70%.

2. Human ^{125}I-HDL was delipidated and passed over Sephadex G-200 to isolate the peptides. Specific activity of apoLP-gln was twice that of apoLP-thr. Additional separation of apoLP-val, apoLP-glu, and apoLP-ala was achieved by DEAE chromatography.

These peptides were individually incubated in human plasma from which the lipoprotein fractions were subsequently isolated. ApoLP-thr and apoLP-glu were recovered with the HDL fraction from which they were originally derived. ApoLP-ala, in contrast, was found evenly distributed in VLDL and HDL (it is normally found in both these fractions). Thus, lipoprotein apoproteins tend to recombine with the plasma lipoproteins in which they are originally located. When the plasma decay of apoLP-thr and apoLP-gln was compared with that of whole human HDL in the rat, the half-life was about 12 hours.

3. Animal Studies -- Rat: Several substrates including iodinated VLDL, LDL and HDL plus $^{125}I^-$ and Cr^{54}-tagged rat erythrocytes were injected intravenously. Plasma decay and tissue radioactivity were measured. Tissue levels varied with different substrates. Inorganic ^{125}I was secreted rapidly in urine, bile, and seminal fluid resulting in increased activity in kidneys, intestinal contents, intestinal wall and testes. Only small amounts were in other tissues. Most Cr^{54} activity was found in the spleen. Upon injection of ^{125}I lipoproteins, specific activity and total activity was greatest in the liver. Hepatic uptake of LDL and HDL radioactivity was comparable in rate, whereas VLDL uptake was much more rapid. VLDL was also shown to undergo rapid conversion to LDL. No tissue appeared to accumulate and retain radioactivity. These studies suggest that the liver plays a major role in lipoprotein catabolism. Furthermore, the lipoprotein protein moiety appears to degrade rapidly following uptake by the cell.

4. Preliminary studies of the effect of carbohydrate feeding on LDL synthesis were conducted in one type II patient. Results indicated a slight increase in synthesis (15%) while he was consuming a high carbohydrate diet, but restriction in fat consumption necessitated by this diet also caused a slight increase in fractional catabolic rate (FCR). More studies in this area are underway.

Significance to Biomedical Research and Program of the Institute: Studies of LDL metabolism have thus far been valuable in indicating the defect in type II hyperlipoproteinemia and have also provided insight into effects of diet and drugs on lipoprotein metabolism. Extension of these studies to the tissue level are likely to provide valuable information regarding determinants of lipoprotein synthesis and degradation. The specific defects that underlie the hyperlipoproteinemias will hopefully be found. This information will be valuable not only in helping design more rational therapy for these disease states but will also aid in understanding the basic processes involved in lipid transport.

Proposed Course:

1. Studies of beta lipoprotein (LDL) turnover will continue, and effects of dietary manipulation and drug treatment on its metabolism will be studied. Specific attention will be given to effects on LDL catabolism during ingestion of pharmacologic doses of chenodeoxycholic acid. Chenodeoxycholate inhibits hepatic bile acid synthesis which is a principal route of cholesterol degradation. Effects of LDL catabolism in this setting will help understand one more possible modifier of LDL metabolism.

2. The metabolic relationships that exist between different lipoprotein classes in all dyslipoproteinemias will continue to be studied.

3. Individual HDL peptides as well as intact HDL will be studied further in animals and humans. Special emphasis will be given to Tangier disease where HDL is deficient.

4. Catabolism of all lipoproteins will remain a major project with most studies being performed in suitable experimental animals, including the rat and monkey.

Publications:

Langer, T., Strober, W., and Levy, R.I.: The metabolism of plasma lipoproteins. In Plasma Protein Metabolism, M.A. Rothschild and T. Waldmann, Editors, New York, Academic Press, 1970.

Levy, R.I., Langer, T.: Hypolipidemic drugs and hyperlipoproteinemia. New York Academy of Sciences (in press), 1971.

Levy, R.I., Bilheimer, D.W. and Eisenberg, S.: The structure and metabolism of chylomicrons and very low density lipoproteins (VLDL). The Biochemical Journal, 1971.

Serial No. NHLI-277(c)
1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: The Metabolism and Structure of Plasma Lipoproteins in the Rat.
Previous Serial Number:	NHLI-302(c)
Principal Investigators:	Robert I. Levy, M. D. Peter Herbert, M. D. Donald S. Fredrickson, M. D.
Other Investigators:	Elanne Smootz, B. S. Barbara Miller
Cooperating Units:	Herbert Windmueller, Ph.D., Section on Nutritional Biochemistry, NIAMD Thomas Bersot, B.A., Virgil LeQuire, M.D. Department of Pathology Vanderbilt University, Nashville, Tennessee

Project Description:

Objectives: Techniques have been developed in the rat for obtaining lipoproteins in the perfusate from isolated liver, plasma, and lymph from the isolated and intact intestine. These systems provide excellent experimental models for studying lipoprotein physiology. They afford particular access to the synthesis and interrelations of the lipoprotein classes and their responses to perturbations. Since the apolipoproteins of the rat had not been as well studied as their human counterparts, effort in the past two years has been directed toward their isolation, fractionation and characterization.

Methods: The methods employed were those outlined in last year's annual report and were similar except in minor detail to those applied to the study of human apolipoproteins. Preliminary experiments with C^{14}-lysine addition to organ perfusates were also performed. The distribution of radioactivity in the apolipoproteins was determined by monitoring the labeled bands in sliced polyacrylamide gels.

Major Findings:

1. The major rat apolipoproteins were purified and characterized as

to their amino acid composition, terminal groups, molecular weight, optical rotatory dispersion and immuno-reactivity, in continuation of studies reported last year.

2. All peptides isolated from VLDL and HDL were evaluated in a lipolytic system for activation of lipoprotein lipase. As in the earlier human studies, only one peptide (V3-3) corresponding to apo-LP-glu was active.

3. C^{14}-lysine proved to be a satisfactory amino acid for following the vivo synthesis of the apolipoproteins. Radioactivity was incorporated primarily into protein with only small amounts segregating with lipids. Preliminary experiments, while not conclusive, suggest that the intestine may not rely exclusively on in situ synthesis to supply apoproteins for chylomicrons.

4. Immunochemical and electrophoretic data were not wholly consistent with the hypothesis that liver perfusate VLDL and intestinal lymph chylomicrons are identical in protein content. Further interpretation must await the isolation and characterization of the apoproteins of chylomicrons.

Significance to Biomedical Research and the Program of the Institute:
The apolipoproteins of the rat differ qualitatively from those of man in their terminal amino acids, amino acid composition and in their quantitative distribution in extra cellular fluid. Nevertheless, there are similarities in their apparent size, charge and number. They promise to serve as a useful model system for unraveling the roles and fate of the human apolipoproteins.

Proposed Course:

1. Techniques similar to those used with plasma will be employed to isolate and characterize the apoproteins of rat intestinal lymph.

2. The incorporation of a radioactive amino acid into apolipoproteins by the isolated perfused liver and intestine will be evaluated and compared to define the principal sites of lipoprotein apoprotein synthesis.

3. Further studies of the carbohydrate content of the rat apolipoproteins are in progress.

Publications:

Bersot, Thomas P., Brown, W. Virgil, Levy, Robert I., Windmueller, H.G., Fredrickson, Donald S and V. S. LeQuire. Biochemistry 9: 3427 (1970)

Serial No. NHLI-278(c)
1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: Studies on a new lipoprotein complex in plasma, its structure, function and genetic control.
Previous Serial Number:	NHLI-301(c)
Principal Investigators:	Robert I. Levy, M. D. Alan K. Rider, M. D. Donald S. Fredrickson, M. D.
Other Investigators:	Elanne Smootz
Cooperating Units:	None

Project Description:

Objectives: Electrophoresis on paper or agarose gel and ultracentrifugation allows the separation of the plasma lipoproteins into four major families; chylomicrons, VLDL (prebeta lipoprotein), LDL (beta lipoprotein) and HDL (alpha lipoprotein). VLDL are characterized by prebeta (or alpha$_2$) migration in electrophoresis and have a density of 1.006 (Sf 20-400). VLDL are composed of peptides having immunochemical similarities to the βLP and αLP as well as several unique peptides. The beta apoprotein (peptide moiety of βLP) comprises about 40% and the α apoprotein about 10% of the total protein of VLDL. The remaining protein is composed of the recently described "D" peptides.

We have recently recognized the occurrence of a fifth major lipoprotein species with prebeta migration on electrophoresis but with a density between 1.050 and 1.085. This lipoprotein appears to be identical to the Lp(a) antigen discovered by Berg. It is the intent of this project to characterize the Lp species and determine the significance of its appearance as "sinking pre-beta" lipoprotein in some subjects.

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Methods: Lipoproteins were obtained from fasting subjects by plasmapheresis and isolated by preparative ultracentrifugation. The density fractions were then further characterized by polyacrylamide gel and paper electrophoresis as well as immunochemical techniques. Totally delipidated apoproteins were obtained by chloroform-methanol (2:1) extractions.

A specific antiserum made to this new lipoprotein was used to screen a population of subjects utilizing two different techniques for immunochemical analysis.

Major Findings:

1. Serum from three thousand five hundred subjects has been studied by staining paper electrophoretic strips with Oil Red O. The Lp lipoprotein has been demonstrated as a distinct pre-β migrating band contained in the fraction heavier than 1.006 gms/ml, in 10.9% of the individuals. This Lp lipoprotein is present in normals as well as subjects with the five major types of hyperlipoproteinemia, Tangier disease and hypobetalipoproteinemia. The presence of high concentrations of Lp is not related to high concentrations of VLDL or LDL.

2. The Lp lipoprotein does not appear to be influenced by diet. Patients have been placed on high and low cholesterol as well as high carbohydrate diets without influencing the amount of Lp as determined by the density of the band on paper electrophoresis stained with Oil Red O. Intravenous injection of heparin does not change the amount of Lp.

3. The presence of the Lp lipoprotein has been investigated using two immunochemical techniques, Ouchterlony double diffusion and counter-electrophoresis (CEP). In 550 samples screened with the Ouchterlony technique, Lp(a) was present in 42%. With the considerably more sensitive CEP technique, the antigen was detected in 77% of 300 samples tested.

4. On electrophoresis in 3% acrylamide, in a standard Tris HCl discontinuous buffer system, the Lp lipoprotein migrates more slowly than βLp, suggesting that the lipoprotein is larger in size than βLp. This is confirmed using 2% agarose chromatography. The Lp lipoprotein and βLp elute in one large peak, but the Lp is on the upslope whereas the βLp is on the downslope of the peak.

5. Heparin-manganese precipitates the Lp lipoprotein as well as βLp.

6. Isolation of the Lp lipoprotein has been achieved using the principles of affinity chromatography. The specific antiserum is bound to 4% agarose by the cyanogen bromide method. A serum fraction isolated in the ultracentrifuge between density 1.019 and 1.125 gms/ml is then passed over the column in 0.02 M Tris HCl, pH 7.8. The βLp and HDL_2 (αLp_1) elutes while the Lp forms an antigen antibody complex on the column. The Lp is then eluted with 0.1 M NaOH, pH 12.8. This fraction is contaminated with a small amount of αLp. The αLp is separated from Lp by passing the fraction over a 2% agarose column equilibrated in 0.1 Tris HCl, pH 7.8.

7. The lipoprotein has been delipidated with chloroform-methanol (2:1). 34.9% of the complex is protein, 7% triglyceride, 29.6% cholesterol, and 28.5% phospholipid. The protein component can be solubilized by the addition of 0.01 borate buffer, pH 11.5, 8M urea or with 100 millimolar decyl sulphate.

Significance to Biomedical Research and the Program of the Institute:

The nature and function of Lp is still unknown. It may be a form of β-lipoprotein and conceivably may have some relationship to vascular disease. Further exploration is highly indicated.

Proposed Course:

1. The discrepancy between the presence of the Lp antigen in serum using the double diffusion and counter-electrophoretic technique must be explained. It has been assumed by Berg that the antigen is inherited as an autosomal dominant trait. Although the data from our studies of paper electrophoresis and double diffusion clearly support the inheritance of the trait, the incidence appears to be much higher; perhaps 80% of the population has Lp at least in very low concentration. We will attempt to prove with more sensitive techniques that the lipoprotein may be present in all sera and that the presence of higher levels may be an expression of a genetic trait.

2. We have perfected a technique for isolating the Lp lipoprotein devoid of contamination with βLp and αLp by combination of affinity and molecular-sieve chromatography.

Reliable information can now be obtained as to the lipid composition as well as the solubility characteristics, amino acid analysis, secondary structure and antigenetic characteristics of this unique apoprotein.

Publications:

None other than abstracts.

Serial No. NHLI-279(c)
1. Molecular Disease Branch
2. Section on Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: Post-heparin lipolytic enzymes and their role in normal and abnormal lipid transport and clearance.
Previous Serial Number:	NHLI-304(c)
Principal Investigators:	Robert I. Levy, M. D. Ronald Krauss, M. D. Donald S. Fredrickson, M. D. Peter Herbert, M. D.
Other Investigators:	Laura Miller, B. A.
Cooperating Units:	S. Baylin, M. D. Experimental Therapeutics Branch, NHLI John LaRosa, M.D. Div. of Community Medicine, George Washington Medical School

Project Description:

Objectives: The metabolic breakdown and interconversion of plasma lipoprotein is as yet incompletely understood. It has been postulated that in the process of transferring circulating triglyceride from plasma to tissues, the lower density lipoproteins (chylomicrons and VLDL) are converted to progressively higher density lipoproteins by a series of lipolytic reactions. Heparin injection is known to release into plasma a number of lipolytic enzymes (collectively referred to as post-heparin lipolytic activity or PHLA) which are thought to play a major role in this process. In man, a deficiency state of one or more of these enzymes is characteristic of at least one of the genetically determined hyperlipidemic states (Type I hyperlipoproteinemia).

The aim of this project is three-fold:

1. To characterize further the heparin released triglyceride lipase (TGL) in animal tissues and in human post-heparin plasma.

2. To determine the nature of possible enzymatic abnormalities in various disorders of lipoprotein metabolism.

3. To purify the triglyceride lipase or lipases from post-heparin plasma.

Methods: Pre- and post-heparin plasma was obtained from normal subjects and from patients with familial hyperlipoproteinemia on a variety of diets. Samples were obtained at variable intervals (usually 10 minutes) after standard doses of intravenously administered heparin (10 u/kg or 75 u/kg). Acetone-ether powders of rat adipose tissue and liver were also used as enzyme sources.

TGL activity was assayed using pure ^{14}C-glyceryl triolein as substrate, sonified in tris buffer at pH 8.6 containing albumin, Triton detergent, and sodium chloride. Fatty acid released during lipolysis was isolated from glyceride by extraction into hexane and then into 0.1Mpotassium hydroxide. Labelled fatty acid was then measured by counting in a liquid scintillation spectrometer.

Attempts to purify TGL from post-heparin plasma utilized affinity chromatography on solid supports containing covalently linked heparin.

Major Findings:

1. While it has been known for some time that TGL required HDL as a co-factor, the exact nature of the co-factor has remained obscure. Using rat adipose tissue powders as enzyme source, various purified lipoprotein peptides were screened for co-factor activity. Two peptides which are minor constituents of HDL and major constituents of VLDL, were shown to have significant co-factor activity, whereas the other peptide components of HDL and VLDL were inactive. One of the active peptides is known to contain carboxy-terminal glutamic acid; the other is as yet uncharacterized. Both peptides show enhanced co-factor activity in the presence of phospholipid, although phospholipid is not essential for activity. The results suggest that these peptides normally function to facilitate the hydrolysis of VLDL by lipoprotein lipase.

2. The defect in lipoprotein metabolism in Type I hyperlipoproteinemia has been further investigated. One of the hypotheses that has been advanced is that Type I patients are able to synthesize a normal enzyme molecule, but lack the necessary activating mechanism. This theory was tested using the information already obtained regarding the identity of the activating peptides in non-Type 1 individuals. Two Type I patients were found to have normal quantities of both activating peptides previously described. Each of the peptides was as potent in activating rat adipose TGL as its counterpart in normal individuals. Furthermore, addition of activators isolated from a Type V patient failed to enhance Type I PHLA activity. These data strongly suggest that the abnormality in Type I does not involve the activating system.

3. Another approach to the elucidation of the defect in Type I was undertaken in collaboration with Dr. S. Baylin of the Experimental Therapeutics Branch. A diamine oxidase known as histaminase has been shown

to be released into plasma by heparin in a manner similar to PHLA. Patients with various forms of hyperlipoproteinemia were screened for post-heparin histaminase activity. Most patients fell within the normal range, but those with Type I had virtually absent activity even after receiving maximal amounts of heparin. To investigate the possibility that histaminase and PHLA are in fact the same enzyme, the two enzyme assays were conducted in the presence of specific inhibitors; histaminase was not inhibited by protamine, and PHLA was not inhibited by aminoguanidine and histamine, both of which are potent inhibitors of histaminase. Finally, the possibility that Type I plasma contains a histaminase inhibitor was ruled out by demonstrating that addition of Type I to normal post-heparin plasma did not alter the normal histaminase activity. These data suggest that the abnormality in Type I patients may involve a tissue binding site or a release mechanism (in common with histaminase) rather than a defect in enzyme production or function.

4. The presence of variable quantitites of endogenous substrate (chylomicrons and VLDL) in post-heparin plasma creates difficulties in standardizing the PHLA assay. This problem has been overcome by providing high levels of triolein in the assay medium. Using a concentration of 7.5 mg triolein per cc of assay, PHLA was measured with a high degree of sensitivity and reproducibility irrespective of plasma triglyceride levels. The enhanced sensitivity of this radioactive assay has made it possible to use small quantities of PHLA (25-50 microliters), thus further reducing the amount of endogenous triglyceride added to the substrate mixture. This system has been shown to follow zero-order kinetics for up to three hours at 27°C and for at least one hour at 37°C. It is suggested that the enhanced stability of the enzyme to heat inactivation when compared with other assay systems is related to the presence of added substrate.

5. Initial efforts at purification of TGL post-heparin plasma have utilized heparin covalently linked to either agarose or polyacrylamide gel. PHLA can be absorbed onto a column made of this material, and can be eluted in a partially purified form with solutions of variable pH and ionic strength.

Significance to Biomedical Research and the Program of the Institute:

The enzymes involved in degradation of very low density lipoproteins and chylomicrons have great relevance to disorders associated with abnormal concentrations of the plasma lipids and the increased incidence of atherosclerotic vascular disease.

Proposed Course:

1. The nature of the TGL co-factor will be further investigated by more precise biochemical definition of the active site, and by inquiry into the kinetics of activation.

2. The effects of substrate composition on adipose and plasma TGL will be investigated, particularly with respect to the role of phospholipids and cholesterol.

3. Attempts will be made to differentiate the individual enzymes present in PHLA both by alteration of assay conditions, and by physical separation methods. Using these techniques, it is hoped that the abnormalities in the various forms of hyperlipoproteinemia may be better delineated.

4. By further screening, the specificity of the post-heparin histaminase assay in differentiating type I from other types of hypertriglyceridemia will be evaluated.

Publications:

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Serial No. NHLI-280(c)
1. Molecular Disease Branch
2. Sections on Lipoproteins and Molecular Diseases
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: Studies of familial hyperlipoproteinemia
Previous Serial Number:	NHI-307(c)
Principal Investigators:	Donald S. Fredrickson, M. D. Robert I. Levy, M. D. David W. Bilheimer, M. D. John Brensike, M. D. Jan Breslow, M. D. Shlomo Eisenberg, M. D. Antonio Gotto, M. D.,, D. Phil. Peter N. Herbert, M. D. Antonio Jover, M. D. Samuel Lux, M. D. Alan K. Rider, M. D. Richard S. Shulman, M. D. Howard R. Sloan, M. D., Ph.D. Neil J. Stone, M. D.
Other Investigators:	Better Masket, B.S., M.A. Nancy Mason, B.A. Marguerite LaPiana, B.A., Nancy Priddy Margaret Hill Kathryn John, B.A.
Cooperating Units:	Stephen Epstein, M. D., Diagnostic Cardiology Branch, National Heart and Lung Institute Nancy D. Ernst, B.S., Merme Bonnell, B.S., M.S., and Edith Jones, B.S., M.S., Nutrition Department, Clinical Center Joan Gurian, B.S., Biometrics Branch, National Heart and Lung Institute

Project Description:

Objectives: This is the largest single project devoted to clinical investigation in the laboratory. Its aim is to classify, determine the number of responsible mutations, the nature of underlying mechanisms, better define the clinical manifestations and prognoses and develop treatment for familial excesses in blood lipid concentrations. Over six years ago a system of methodology and nomenclature for the lipid transport disorders was introduced as a standard technique for phenotyping or differentiating the hyperlipoproteinemias that would have wide practical applicability to both the clinician and the geneticists. The system has been undergoing intensive testing and refinement since that time. The objectives sought with the use of this system have been fully outlined previously. They are in brief: (1) proof that lipoprotein patterns provide more useful information than determination of lipid concentrations alone, (2) development of an analytical sequence beginning with a simple technique capable of rapidly and economically separating "normals" from "abnormals" and capable of further dividing the latter into "Types" indicating different groups of metabolic abnormalities, (3) application of the analysis to as large and representative number of patients and their families as is possible, (4) correlation of these analyses with other clinical and genetic features to further test the assumption that lipoprotein patterns are capable of distinguishing different phenotypes, and (5) expansion of techniques for determining genotypes and the basic metabolic abnormalities.

The first two objectives have been reached. Application has now proceeded to samples from over ten thousand patients.

Methods: With a large staff of physicians and automated methods for accurately determining plasma triglyceride and cholesterol concentrations coupled with twelve Durrum electrophoretic cells the laboratory now has the capacity for screening and evaluating over 1,000 samples per month.

Samples are accepted from donors referred locally and throughout the nation. Patients are seen in a weekly clinic that has now expanded so as to average some 30 to 50 individual patient visits weekly. Certain selected patients with familial hyperlipoproteinemia are accepted for inpatient or outpatient metabolic studies predominately drug or dietary of from one to 200 weeks duration. The basic technique includes the paper electrophoretogram, employing albuminated buffer. All samples are sent through cholesterol and triglyceride determinations and all are examined after standing in the cold for 24 hours for the presence or absence of turbidity and/or a discrete cream layer. This permits preliminary segregation of normal and abnormal patterns identified as Types I through V. Selected samples are put through quantitative lipoprotein separation consisting of centrifugation at plasma density, examination of electrophoretic mobility of lipoproteins in supernatant and infranatant fractions, precipitation of all lipoproteins but high density lipoprotein, and separate quantification by cholesterol content of alpha, beta, and very low density lipoproteins. While patients are on diets high in carbohydrate, glucose

tolerance tests with an associated immunoreactive insulin assay is done. Post-heparin lipolytic activity (PHLA) is measured on selected samples as is the activity of post-heparin plasma monoglyceride hydrolase and triglyceride lipase. Many patients are tested for their responsiveness to five basic isocaloric diets: ad libitum, a "balanced" 40-40-20 diet (percent of calories from fat, carbohydrate, and protein) containing either 300 mg of cholesterol with a normal P/S ratio or less than 300 mg of cholesterol with a P/S ratio of 2, and diets high in fat or carbohydrate each with a constant low cholesterol content. Often for both diagnosis and management patients are placed on hypocaloric diet regimens.

Every effort is made to sample all the possible blood relative of a patient and to classify them also into one or more types. Often these samples are obtained from physicians or require field collections.

With the collaboration of the Biometrics Branch, National Heart Institute, all of the information obtained by history, physical examination and laboratory evaluation on inpatients and outpatients has been successfully transposed onto IBM punch cards. This process has now allowed us to put all our patient data in card and tape form with ready availability for computer analysis.

Cholestyramine (Questran^{R}, 16 to 32 grams per day) is now being evaluated in patients with Type II hyperlipoproteinemia, both on open single blind studies on inpatients and in a double blind study with two control periods of 4 weeks, alternating with two periods of placebo and cholestyramine therapy. In selected patients responsiveness to cholestyramine and clofibrate have been compared. An increasing number of patients with Type II have been placed on long-term cholestyramine.

Nicotinic acid as well as the progestational compound norethindrone acetate has been evaluated in selected patients with different types of hyperlipoproteinemia. When possible, these agents' efficacy was compared to nicotinic acid and cholestyramine.

Major Findings:

1. Family studies have now been extended to more than 3,500 patients with classification of some members in approximately 450 different kindreds with familial hyperlipoproteienmia. These include: 18 patients from 12 kindred with Type I, 650 patients from 230 kindred with Type II, 87 patients from 69 kindred with Type III, 178 patients from 86 kindred with Type IV and 76 patients from 37 kindred with Type V. Over 2,500 other abnormal patient samples have been screened, but because of the presence of acquired disorders, evaluation went no further. The data continue to indicate that the first three types of hyperlipoproteinemia are fairly homogeneous and distinct. No patients with Type II have appeared in Type III families or vice versa. Type I appears to be transmitted as a simple mendelian recessive trait. The more common Type III has now occurred in families with enough frequency to lead us to question its inheritance as a simple recessive and wonder about its transmission as an incompletely expressed dominant trait. Type II is likely the most common single genetic lipid transport abnormality; distribution of patterns in the kindreds is compatible with a highly

penetrant autosomal domonant. Type IV is perhaps the most common pattern seen in the population at large. It is clearly heterogeneous. Type IV and V are intermingled in kindreds; the latter type, however, appears to be a separate genotype.

2. Improved methods of data transcription and storage have expanded our capacity, but many request for analyses and assistance to physicians cannot be accommodated. Plans are now underway to streamline the data editing system and make final data more obtainable with online computer terminals.

3. Vascular Disease prevalence in Type II Hyperlipoproteinemia. Using retrospective case study methods, members of 120 kindreds with Familial Type II are being contacted. All first degree relatives of propositi, and then all first degree relatives of any subject with familial Type II hyperlipoproteinemia are being included in the study. All patients are interviewed to determine the prevalence of angina pectoris, myocardial infarction, intermittentclaudication, and stroke. Supportive hospital and physician data are being compiled to substantiate rigid diagnostic criteria. The interview also records information on smoking, blood pressure, glucose tolerance, family history of coronary heart disease (CHD) and any therapeutic interventions (diet and/or drug). If the patient is deceased, death certificates are obtained and a family member contacted to determine if death was sudden. Autopsy reports, when available, are obtained. A current 12 channel resting ECG is obtained on all patients in the study if one was not performed since January, 1970. Whenever possible, Beta quantifications have been performed at the NIH to document the presence or absence of increased low density lipoprotein (LDL).

As of March 30th, approximately 40 kindreds have been completely contacted. In an additional 20 kindreds, interviews have been performed on some but not all subjects. In over 100 kindreds, much historical information has been obtained. Information on twenty-four kindreds selected at random at the start of the study is almost complete and soon analysis of this data should give preliminary results.

4. Type II Hyperlipoproteinemia Intervention Study. In cooperation with the Cardiology Branch the Lipid Metabolism Branch has begun a study aimed at elucidating the effect of treatment of Type II on the rate of progression of coronary artery disease. The study is designed to answer the question "Does the reduction of plasma LDL in subjects with Type II hyperlipoproteinemia cause regression or decreased progression of premature coronary artery disease?"

This study will involve enrollment of 250 Type II patients with coronary artery disease from the local area. This group will receive extensive lipid and cardiologic evaluation (including coronary angiography) and will be split into treatment (Type II diet plus 24 gm. of Cholestyramine) and control (Type II diet plus placebo) groups. They will be followed monthly and receive intensive re-evaluation at two and five years including repeat coronary angiography. The end points which will be sufficient to terminate the study prematurely are significant differences between the

treatment and control groups in (1) mortality and new myocardial infarction or (2) rate of progression of coronary artery disease as shown on coronary angiography.

The planning and organization of the study and the testing and acquisition of the necessary associated machinery and materials is nearing completion. It is anticipated that the enrollment of patients for the study will begin on or around July 1, 1971.

5. Evaluation of Different Systems and Techniques for Defining Hyperlipoproteinemia. Attempts have been made to stress the utility of the cholesterol and triglyceride determinations coupled with a view of the plasma as the initial step in lipoprotein phenotyping. In Type I the TG/C ratio is often 10/1 and there is a large discrete cream layer over a clear infranatant plasma. In Type II cholesterol is increased but triglyceride is often normal; the plasma is clear. In Type III cholesterol and triglyceride are often increased to the same extent and depending on the patient's diet the plasma may be clear to grossly turbid with or without a discrete cream layer. In Type IV the plasma cholesterol concentrations are often normal. The triglyceride concentration is always elevated usually in the 300-900 mg% range. Depending on the degree of triglyceridemia the plasma may be clear diffusely turbid or creamy. In Type V triglyceride concentrations are usually grossly increased above cholesterol. The plasma is usually diffusely turbid with a distinct cream layer on top.

In normals and subjects with Types II and IV hyperlipoproteinemia it was found that β-lipoprotein concentrations (in terms of βLP cholesterol) could be accurately estimated if one knows only the plasma and HDL lipoprotein cholesterol concentrations and the plasma triglyceride. Using the formula (β-lipoprotein (LDL) cholesterol = plasma cholesterol - HDL cholesterol - TG/5), LDL concentrations can be accurately determined in all subjects free of chylomicra with triglyceride less than 400 and without the Type III abnormality.

6. Dietary Management of Hyperlipoproteinemia. In collaboration with the Nutrition Department of the National Institutes of Health a dietary handbook for physicians as well as individual diet manuals for each lipoprotein phenotype have been written and revised. The diets were based on our experience with hundreds of patients on the wards and the outpatient clinics of the National Heart and Lung Institute. In Type I a low fat diet (25-35 grams) is described and a special section has been detailed on formula feeding for the very young. In Type II an extremely low cholesterol diet modified in fat (high P/S ratio) is recommended. The diets for Types III, IV and V are broken down into two parts. In step I caloric restriction and reduction to ideal body weight is recommended. In step 2, Type III, the percent of calories from CHO and fat are balanced (40% as each) and cholesterol is restricted. In Type IV carbohydrate and alcohol are modestly restricted. In Type V the dietary fat content is restricted to less than 70 grams per day, protein content is increased and alcohol is eliminated. The diet manuals were written with an attempt at supplying the physician, dietician and patient with useful information and practical effective dietary regimes. In all manuals it is stated that

these diets are not for normal subjects. Thus far, over 250,000 handbooks and manuals have been distributed. Their demand is great.

7. Cholestyramine (Questran ^{R}), has now been given to over 80 patients with familial Type II. In the Type II heterozygote cholesterol can now be brought within normal limits. In some 48 completed double blinded trials with cholestyramine and placebo a mean reduction of cholesterol and βLP cholesterol of 25-40% has been achieved. Inpatient studies (in Type II subjects) have clearly shown that a low cholesterol high P/S diet and cholestyramine in a dosage of 16-24 grams per day are additive and more effective than either drug or diet alone. Resolution of tuberous xanthomas and xanthelasma has now been observed in 8 Type II heterozygotes on long term diet and drug therapy. In Type II cholestyramine has resulted in a minimal but statistically significant increase in plasma triglyceride concentrations. In other types of hyperlipoproteinemias, Type III-IV-V, this triglyceride increase is often striking making the resin therefore only efficacious in Type II.

In 10 Type II homozygotes the combination of cholestyramine and diet have been variably effective. In 3 homozygotes this has resulted in a normalization of blood lipids. In 6 other subjects addition of nicotinic acid to the above regime normalized the blood lipids (3) or at least drastically reduced cholesterol levels to below 400 mg%. In 1 Type II homozygote, age 5, neither cholestyramine (32 grams/day) nor nicotinic acid have effectively lowered the cholesterol.

In a series of patients nicotinic acid (3 grams/day) has proven more effective than clofibrate (2 gms/day) in the management of Type V hyperlipoproteinemia (6 patients). In contrast, in Type II nicotinic acid has lowered cholesterol and βLP concentrations but at rather high dosages (4.5 - 9.0 gms/day).

8. Lipid Research Clinics. The Molecular Disease Branch has collaborated with the newly formed Lipid Metabolism Branch of the Collaborative Studies Program-NHLI to help establish Lipid Research Clinics. The clincs will serve both an educational and research role. They will aim to standardize basic as well as sophisticated lipid and lipoprotein techniques, gather data about the prevalence of hyperlipoproteinemia and through a central registry serve as the foci for future intervention studies.

Significance to Biomedical Research and the Program of the Institute: Successful recording of the concepts and classification of blood lipid abnormalities is of considerable and practical importance to physicians. Some of these disorders play an important role in the determination of the susceptibility to atherosclerosis. Understanding the pathogenesis and mechanisms of abnormal lipid transport as demonstrated in patients with these disorders should hasten the understanding of normal processes and normal susceptibility to vascular disease.

Proposed Course:

1. The objectives outlined in the introduction will continue to be pursued with increased emphasis on determination of abnormal mechanisms. Continued improvements in typing will be sought; the laboratory also instructs many outside laboratories in the techniques. Representatives of well over 200 laboratories have been accommodated during the past year.

2. In collaboration with the Cardiology Branch further objective correlation of hyperlipoproteinemia and coronary and peripheral artery disease will be pursued and short and long term effects of drug and diet therapy on the vascular system will be evaluated. Attempts will continue to be made to establish what proportion of the patients with premature vascular disease and hyperlipoproteinemia have a familial metabolic disorder, and to define both retrospectively and prospectively the true vascular risk of the different types.

3. Evaluation of the efficacy of nicotinic acid, cholestyramine and clofibrate will continue. In those types who have already proven to be nonresponsive to clofibrate or cholestyramine, other lipid lowering agents will be evaluated. Further evaluation of the effects of variation in dietary constituents on the types of hyperlipoproteinemia will continue.

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Serial No. NHLI-281(c)
1. Molecular Disease Branch
2. Section on Lipoproteins and Molecular Disease
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: The Structure of Very Low Density Lipoproteins.

Previous Serial Number: NHLI-305(c)

Principal Investigators: Peter Herbert, M.D.
Robert Levy, M.D.
Donald Fredrickson, M.D.
Howard Sloan, M.D.

Other Investigators: Elanne Smootz, B.S.
Barbara Miller (from April 1)

Cooperating Units: None

Project Description:

Objectives: The very low density lipoproteins (VLDL) are responsible for the transport of most of the body's glyceride from sites of origin to sites of catabolism and storage. The apoprotein moiety of VLDL, while comprising only 10% of the particle mass, appears to play a critical role in the structural integrity of the lipoprotein. Moreover, it seems to contain essential cofactors for the lipoproteins' catabolism. This project is a continuation of work proceeding for the last five years on the quantification and characterization of the apoproteins of VLDL.

Methods: VLDL was prepared from plasma of normal subjects and patients with Types I, III, IV and V hyperlipoproteinemia. The techniques employed were those described in previous annual reports. After delipidation and solubilization the apo-VLDL was fractionated by gel and ion exchange chromatography. The various fractions obtained were analyzed as to their immunochemical and electrophoretic properties, amino acid composition, carbohydrate content and capacity to activate lipoprotein lipase. The techniques used were those previously reported from our laboratory.

Major Findings:

1. It has been possible to refine further the chromatographic techniques for the isolation of the apo-VLDL peptides in homogeneous form. As previously reported, the peptides with COOH terminal serine, glutamic acid, valine and alanine comprise 80-85% of the total VLDL protein. The remaining 15-20% is composed of several peptides which differ in their electrophoretic mobility, chromatographic properties and amino acid composition. They are subject of further analysis.

2. In addition to apoLP-glu, at least one additional peptide representing 2-3% of the total VLDL protein has been found to activate lipoprotein lipase. Its immunological and biochemical properties are under investigation.

3. The carbohydrate composition of some of the major peptides has been quantified. ApoLP-val contains no carbohydrate. ApoLP-ala is present in two forms. On a molar basis one form contains N-acetyl neuraminic acid (NANA): galactose: galactosamine in a 1:1:1 ratio. The other form is identical except for the presence of 2 moles of NANA per mole of protein. ApoLP-glu contained no sialic acid and galactose, galactosamine and glucosamine were present in less than molar quantities. The latter findings are difficult to interpret and additional determinations are necessary.

4. With improved techniques, the relative apoprotein composition of VLDL in four of the five types of hyperlipoproteinemia was evaluated. No defect in the quality or relative quantity of the major VLDL apoproteins was found. ApoLP-glu obtained from the VLDL of Types I, III, IV and V has been found to activate lipoprotein lipase.

Significance to Biomedical Research and the Program of the Institute:
All of the major and several minor apoproteins of plasma VLDL have been isolated in quantity in homogeneous form. Detailed analysis of their structure has been undertaken. A functional role has been found for at least two of them (lipoprotein lipase activation). It will now be possible to evaluate in detail their peculiar affinity for lipids. The structure and function of these lipid binding proteins is particularly germane to the study of vascular disease.

Proposed Course:

1. Studies of the primary secondary and tertiary structure of the VLDL peptides will be continued.

2. Systems will be established for investigating the interaction of these peptides with various lipid combinations.

3. The role of carbohydrate in determining the immunological and physical-chemical properties of these peptides will be further investigated.

4. The protein composition of chylomicrons will be evaluated in detail.

Publications:

LaRosa, J.C., Levy, R.I., Herbert, P., Lux, S.E., and Fredrickson, D.S.: A specific apoprotein activator for lipoprotein lipase. Biochem. Biophys. Res. Comm. 41:57, 1970.

Brown, W., Virgil, Levy, Robert I., and Fredrickson, Donald S.: Further characterization of apolipoproteins from the human plasma very low density lipoproteins. J. Biol. Chem. 245: 6588, 1970.

LaRosa, J.C., Levy, R.I., Brown, W.V., and Fredrickson, D.S.: Changes in high density lipoprotein protein composition after heparin-induced lipolysis. Am. J. Physiol. 220: 785-791, 1971.

Serial No. NHLI-282(c)
1. Molecular Disease Branch
2. Sections on Protein Structure and Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Biochemistry and Metabolism of Plasma Lipoproteins: The structure and genetic control of lipoproteins

Previous Serial Number: NHLI 311(c)

Principal Investigators: Antonio M. Gotto, Jr., M.D., Ph.D.
Robert I. Levy, M.D.
Donald S. Fredrickson, M.D.

Other Investigators: Mariel Birnbaumer, Ph.D.
Samuel Lux, M.D.
Ronald Hirz, M.D.
Sumana Devi, Ph.D.
Richard Shulman, M.D.
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Cooperating Units: Tom Bersot, B.A.
Vanderbilt School of Medicine
Nashville, Tenn.

William Bean, Ph.D.
General Electric Corp.
Schenectady, New York

Project Description:

Objectives: The major objectives of this project have been the study of the physical and chemical determinants of lipid transport by the plasma lipoproteins and of the possible relationship of these determinants to lipid transport disorders. The main areas of investigation have included the physical and immunochemical properties of apoLDL, (including the development of a specific radioimmunoassay), the use of electron spin resonance to probe lipid protein interactions, the application of laser beam spectroscopy to the quantification and characterization of the plasma lipoproteins, the development of theoretical models for representing lipoprotein structure and the characterization of rat plasma lipoprotein--proteins.

Methods: Procedures for the isolation of the plasma lipoproteins and their apoprotein moieties are as described in a previous report [NHI-311(c), 1969-1970]. Methods for the preparation of spin-labeled lipoproteins and apoproteins with N-(1-oxyl-2,2,6,6-tetramethyl-4-piperidinyl)-maleimide were also described in this earlier report. Physical probes used to examine lipid-

protein interactions were electron spin resonance (ESR), circular dichroism (CD) and laser beam spectroscopy. Immunological methods employed included immunoelectrophoresis, double diffusion on Ouchterlony plates and radioimmunoassay.

Major Findings:

1. Structure and properties of the apoprotein moiety of LDL.

a) Method of preparation of apoLDL. LDL was delipidated with chloroform: methanol (2:1) or ether:ethanol (3:1). The former procedure yielded an apoprotein with a phospholipid content of <01% (mg phospholipid per mg protein) while the latter method gave a product containing 1 to 2% phospholipid. The apoproteins were then solubilized by either incubation at pH 11.0 or by treatment with 100 mM sodium decyl sulfate as previously described. The method of delipidation or of solubilization did not alter the immunochemical properties of the apoprotein as judged by double diffusion experiments or by radioimmunoassay. Nor was the conformation of apoLDL, as determined by circular dichroism (CD), affected by the method of preparation, if the spectrum was measured at pH 8.0. At pH 11.0 apoLDL didplayed the CD spectrum expected of a disordered protein. Adjustment of the pH to 8.0 effected an immediate change to a spectrum characteristic of β-structure.

b) Apoprotein heterogeneity of LDL. When isolated between densities 1.019 to 1.063, apoLDL was found by polyacrylamide gel electrophoresis (PGE) and by double diffusion Ouchterlony experiments to contain as much as 5% of the major apoproteins found in VLDL, i.e., apoLP-ala, apoLP-val and apoLP-glu. The content of these three proteins could be significantly reduced by isolating LDL between the more narrow density range of 1.025 to 1.050.

c) Development of a radioimmunoassay for apoLDL. Anti-apoLDL serum was prepared by injection of human apoLDL into rabbits. The globulin fraction containing the anti-apoLDL activity was precipitated with Na_2SO_4, dialyzed against saline and bound to bromoacetylcellulose by a published procedure. The insoluble antibody was able to precipitate 95% of added I^{125}-labeled LDL. Assays were performed in 0.05 M borate buffer, pH 8.5, containing 3% human albumin. The incubation mixture contained 300 µl of the albuminated borate buffer, 100 µl of I^{125}-LDL, 15 µl of suspension of the bound antibody and varied quantities (up to 100 µl) of the unknown sample to be assayed. Incubation was carried out at 4°C for 20 h with constant shaking. The mixture was then centrifuged for 30 min at 2000 rpm at 4°C. Both the supernatant (containing free tracer) and the precipitate (containing bound tracer) were counted and a ratio of B/F (bound/free) was calculated. Standard curves were run containing 10 to 1280 nanograms of LDL protein. The minimal amount detectable was in the range of 5 to 10 nanograms. On the basis of weight of protein, LDL was 3 to 5 times as effective as apoLDL in the displacement of I^{125}-LDL. ApoLDL and its protein counterpart isolated from VLDL were shown to have indistinguishable displacement curves. This method is from 100 to 1000 times more sensitive than the previous procedures for quantification of LDL-protein.

2. Use of electron spin resonance to study lipid-protein interactions in the plasma lipoproteins. In the previous report, preliminary experiments were described in which the physical probe of electron spin resonance (ESR) was

used to study the structure of lipoproteins to which a stable nitroxide free radical had been covalently attached. It was shown that spin-labeled HDL contains two types of signal; a broad one indicating strong immobilization of the label and a narrow one indicating a weak immobilization. Spin-labeled apoHDL exhibited almost exclusively the narrow type of signal. We have continued this investigation and have used spin-labelling to study relipidation. It has been possible to regenerate the broad signal, characteristic of native HDL, by reconstitution of spin-labeled apoHDL with phospholipid and either triglyceride or cholesteryl ester. Neutral lipid was obligatory for restitution of the broad signal and the requirement could not be fulfilled by unesterified cholesterol. The relative ratio of broad to narrow signals was greatly increased at high ionic strength, suggesting that hydrophobic interactions may be important in immobilizing the covalently-bound spin-label. Addition of ascorbic acid abolished by the broad and narrow signals at essentially identical rates, demonstrating that the label producing each type of signal has equal accessibility to water soluble agents. It was concluded that this technique offers a sensitive indicator of the process of relipidation of apoproteins.

3. Application of laser beam spectroscopy as a structural probe and a method of quantification of the plasma lipoproteins. The spectrum of monochromatic light scattered by a suspension of particles gives information about the motion of the particles and can be used to calculate their translational diffusion constant. In the technique of self-beat spectroscopy, light from a Helium-Neon laser, producing at least 1 mW of power, is focussed onto a cell containing a dilute aqueous solution of the lipoprotein. Each illuminated particle scatters light if its index of refraction differs from that of the surrounding medium. The light scattered into a small range of angles about a given angle is collected onto the photosurface of an RCA photomultiplier tube, producing a current that is proportional to the intensity of light falling on the photocathode. This intensity is constantly fluctuating around its average value due to the Brownian motion of the particles. The temporal fluctuations in the output of photocurrent are a measure of the random motion of the particle and are mathematically related to the translational diffusion constant. We have used this method to measure the diffusion constants of LDL and HDL and the particle size with a precision of 3%. The values obtained for the diffusion constants are in excellent agreement with those obtained by ultracentrifugation.

With the use of optical heterodyne spectroscopy and an autocorrelation technique it is possible to obtain a frequency distribution and a quantification of a mixture of particles based on differences in the size, density and refractive indices of the families of particles. The procedure is referred to as a resistance fluctuation technique and is similar in principle to the Coulter procedure for sizing and counting red cells. Application of the methodology to the quantification of the families of plasma lipoproteins in mixtures and in serum is now in progress.

4. Development of a model for studying induced perturbations of LDL stricture. Electron microscopic studies of plasma LDL have been interpreted to indicate the presence of a dodecahedral arrangement of subunits or of icoshedral symmetry. We have used the dodecahedral structure to develop a computer simulated model, employing thermal motion probability ellipsoids of the twenty

protein subunits. The inputs consist of positional parameters and temperature parameters which specify how the subunits are to be represented on a stereoscopic diagram. The program produces stereoscopic illustrations which aid in the visualization of complex packing arrangements of the subunits and of thermal motion patterns. Interunit distances, angles between units and principal axes of thermal motion are also calculated. By reorientation of the arbitrary orthogonal vectors or by altering the distances between the subunits, it is possible to induce structural distortions and changes in the patterns of thermal motion. It is possible by induced distortions to derive the configuration with the minimal free energy conditions for stability. This mathematical approach will be used as a theoretical basis for the interpretation of electron spin resonance spectra obtained from metal complexes of LDL. It may also be used as a basis of comparison with the distribution of particle density observed in electron micrographs.

5. Study of rat plasma lipoproteins. In collaboration with Mr. Tom Bersot of Vanderbilt University, these studies have continued. Individual apoproteins of VLDL, LDL and HDL have been isolated and compared with respect to absorption spectroscopy, circular dichroism, molecular weight (by ultracentrifugation), immunochemistry, amino acid analysis and activation of lipoprotein lipase. By comparing these properties, it has been possible to gain information about the interrelation between VLDL, LDL and HDL in the rat.

Significance to Biomedical Research and the Program of the Institute. An understanding of the basic mechanisms of lipid transport is essential for the unraveling of the relationship between lipid transport and the pathogenesis of cardiovascular disease.

Proposed Course:

1. Fractionation of the protein of LDL into its polypeptide constituents will be attempted. Isolated polypeptide constituents will be degraded by chemical and proteolytic agents and fractionated by gel-filtration and ion-exchange chromatography. The isolated peptides will be studied for ability to bind lipid, for conformational properties and for immunological activity. In this way it is hoped to identify the specific peptide segments which determine these properties.

2. The radioimmunoassay of LDL will be applied to the quantification of apoLDL in the LDL and VLDL in clinical disorders and to the assay of this protein in the arterial wall.

3. Electron spin resonance will be used to study the symmetry of lipoproteins and the spatial arrangement of the individual constituents. Attempts will be made to test mathematical models of lipoprotein structure by studying the effects of specific perturbations on the ESR spectra.

4. Laser beam spectroscopy will be used to probe lipoprotein structure and will be applied to the problem of quantification of the families of plasma lipoproteins.

5. These studies will be pursued in part at the NHLI and in part in the Division of Atherosclerosis and Lipoprotein Research at Baylor College of Medicine, The Methodist Hospital, Houston, Texas.

Publications

1. Gotto, A.M., Kon, H., and Birnbaumer, M.E.: Electron spin resonance studies of lipid-protein interaction in human serum lipoproteins. Proc. Nat. Acad. Sci. 65: 145-151, 1970.

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3. Gotto, A.M., and Kon, H.: Observations on the conformation of human serum high density lipoproteins using infrared spectroscopy, circular dichroism and electron spin resonance. Biochemistry 9: 4276-4283, 1970.

4. Fredrickson, D.S., Gotto, A.M., and Levy, R.I.: Familial alpha and beta lipoprotein deficiency. In The Metabolic Basis of Inherited Disease, J.B. Stanbury, J.B. Wyngaarden, and D.S. Fredrickson (editors), McGraw-Hill, New York, 1971 (in press).

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Abstracts:

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3. Gotto, A.M. and Kon, H.: ESR spectra of spin-labeled human plasma lipoproteins. Biophysical Society Abstracts 10: 244a, 1970.

4. Gotto, A.M., Levy, R.I., John, K., and Fredrickson, D.S.: Studies on the inherited protein defect in abetalipoproteinemia: The occurrence of very low density low proteins. J. Clin. Invest. 49: 37a, 1970.

5. Levy, R.I., Langer, T., Gotto, A.M., and Fredrickson, D.S.: Familial hypobetalipoproteinemia, a defect in lipoprotein synthesis. Circulation 42, Supplement III: A27, 1970.

6. Hirz, R., Lux, S., and Gotto, A.M.: Lipid-protein interactions in native and reconstituted high density lipoprotein studied with a spin label. Circulation 42, Supplement III: A39, 1970.

7. Lux, S.E., Hirz, R., Shrager, R.I., and Gotto, A.M.: Lipid induced structural changes in human plasma high density lipoproteins. Abstracts of the Biophysical Society, 1971, (in press).

8. Gotto, A.M., and Lux, S.E.: Influence of apoproteins on lipoprotein structure and lipid transport. J. Amer. Oil Chemists Society, 1971 (in press).

Serial No. NHLI-283(c)

1. Molecular Disease Branch
2. Section on Protein Structure and Lipoproteins
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	The Biochemistry and Metabolism of Plasma Lipoproteins: The Structure and Genetic Control of Alpha Lipoprotein.
Previous Serial Number:	NHLI - 299(c)
Principal Investigators:	Samuel E. Lux, M. D. Donald S. Fredrickson, M. D. Robert I. Levy, M. D. Antonio M. Gotto, M. D.
Other Investigators:	Ronald Hirz, M. D. Kathryn John, B. A. Silja Meret, B. A.
Cooperating Units:	Alexander Nichols, Ph.D. Trudy Forte, Ph.D. Elaine Gong, B. A. Donner Laboratories, University of California, Berkeley, California Fred S. Rosen, M. D. Harvard Medical School, Boston, Mass. Robert Skarnes, Ph.D. Worchester Foundation for Experimental Biology, Shrewsbury, Mass. Sidney Fleischer Vanderbilt University, Nashville, Tenn. Richard I. Shrager, Division of Computer Research and Technology, N. I. H.

Project Description:

Objectives: The primary objectives have been the elucidation of the structure and function of normal plasma high density lipoprotein (HDL) and its alteration in Tangier disease, an inherited error of HDL metabolism. As noted in previous reports, an understanding of the pathogenesis of Tangier disease promises to increase our insight into the role of HDL in normal lipid transport. In addition, knowledge of the molecular nature of the lipid-protein interaction in HDL is applicable to the structure of membrane lipoproteins as well as other plasma lipoproteins.

Methods: Plasma was collected by plasmaphoreses from fasting normal subjects and patients with Tangier disease or their relatives. Lipoproteins were isolated between densities of <1.006 (VLDL), 1.006-1.019, 1.019-1.063 (LDL), 1.063-1.210 (HDL), 1.063-1.125 (HDL_2), 1.125-1.210 (HDL_3) or >1.210. Apoproteins were obtained by delipidation with ethanol-ether (1:3) and fractionated by chromatography on G-200 Sephadex or DEAE cellulose in Tris buffers containing 6M urea.

Major Findings:

1. Further Characterization of apo-HDL Subunits: Isolation and preliminary characterization of the two major (R-thr and R-gln) and three minor (R-val, R-glu and R-ala) apoprotein subunits of HDL were reported in detail in last year's report. These studies were continued with particular emphasis on the structure of R-gln. The amino acid composition of R-gln was determined after hydrolysis for 24, 48, 72 and 96 hours, hydrolysis in the presence of β-mercaptoethanol and hydrolysis following performic acid oxidation. No tryptophan, histidine, arginine or cysteine was present. One disulfide bond was found and there were 2 moles of isoleucine and methionine/15,000 gm of protein. C-terminal analysis with carboxypeptidase yielded glutamine. The N-terminal residue was not detectable by the dansyl chloride or phenylisothiocyanate techniques and is presumably blocked. Extensive testing was done to determine the optimal conditions for cleavage by CNBr. The cleavage products were separated by chromotography on Sephadex G-75 and by rechromatography of incompletely fractionated peaks on CM-cellulose. Four fractions, labelled C1, C2, C3 and CX were obtained. Preliminary experiments using the α-hydroxybutyric dehydrogenase inhibition assay described below suggested that C1, C2 and C3 could bind phosphatidyl choline (PC) and that CX had no affinity for PC. Further characterization of these fragments and their lipid binding properties is under active investigation at the present time.

2. Lipid Affinity of ApoHDL R-thr and R-gln and Lipoprotein Reconstitution: ApoHDL will recombine spontaneously with PC but not with free cholesterol (FC), cholestryl ester (CE) or triglyceride (TG), other lipids found in native HDL. Complete relipidation may be effected by sonification of apoHDL and PC in the presence of one or more of the above lipids at a temperature above the liquid-crystalline transition of the lipids used. This technique, developed by Dr. R. Hirz, was used to study the effects of relipidation on the structure of apoHDL as described below.

Several observations suggested that R-gln had a greater lipid affinity than R-thr. In recombination experiments R-gln consistently combined with PL and CE more readily than R-thr. In addition, studies reported last year on the dehydration of HDL indicated that lipid-free R-thr dissociated readily from HDL while R-gln retained a complement of lipids similar to native HDL. In collaboration with Dr. Sidney Fleischer, Vanderbilt University, we tested the ability of R-thr, R-gln and apoHDL to inhibit the recombination of inactive apo-β-hydroxybutyric dehydrogenase with lecithin necessary to produce an active enzyme. Less R-gln (15 µg) than R-thr (57 µg) or apoHDL (40 µg) was required to inhibit the enzyme system by 50%. Similarly, the

maximal inhibition by R-gln (81%) was greater than R-thr (52%) or apoHDL (71%).

3. Circular Dichroism (CD) or Relipidated Apoproteins: The effect of lipid recombination on the secondary and tertiary structure of apoHDL, R-gln and R-thr was monitored by CD measurements in the near and far ultraviolet (UV). Spectra were analyzed by comparison with model spectra of poly-L-lysine using the Modelaide curve analysis program developed by R. I. Shrager. By CD criteria native HDL contained 70% α-helical, 15% beta and 15% disordered structure. Delipidation decreased the helical content by 20% with a corresponding increase in disordered structure. R-thr had more ordered structure (55% helix) than R-gln (35% helix). The effects of organic solvents and urea on the CD spectra of these proteins were completely reversible. Reconstitution of R-thr, R-gln and apo HDL with PC + CE restored, respectively 110%, 80% and 100% of the helical structure of the parent HDL. PC alone restored 50-70% of the increase produced by PC + CE. Spectra of HDL in the near UV were substantially altered by delipidation with a generalized decrease in ellipticity. PC partially, and PC + CE completely restored the near UV spectra to that of native HDL. These studies indicated that both PC and CE are required for complete reorganization of the secondary and tertiary structure of HDL.

4. Electron Microscopy (EM) of Relipidated Apoproteins: In collaboration with Drs. T. Forte and A. Nichols, University of California, we examined the influence of relipidation on the EM appearance of R-thr and R-gln. The apoproteins alone appeared as fine granular aggregates below the resolving power of the microscope. PC alone or PC + FC produced the characteristic "liposomes" or "myelin figures" reported by others. Either R-thr or R-gln when recombined with PC alone or PC + FC produced disks, 55 A thick and 150-200 A wide, which stacked in rouleaux. Similar forms have previously been seen in HDL from lecithin-cholesterol acyl transferase (LCAT) deficient patients and in the d. 1.063-1.210 fraction from dehydrated-rehydrated HDL. All these systems have in common a decreased (or absent) CE content. Completely relipidated R-thr or R-gln, produced by sonification of apoproteins +PC+FC+CE or treatment of apoproteins +PC+FC with LCAT, appeared as round forms with definite subunit structure, similar to native HDL.

5. Endotoxin - Inactivating Activity of HDL: In collaboration with Drs. F. S. Rosen, Harvard Medical School and R. Skarnes, Worchester Foundation for Experimental Biology, we have examined the endotoxin-inactivating properties of HDL. This activity is thought to require at least two steps. One of these is an esterase, confined to HDL, which is assayed with an artificial substrate, β-napthyl acetate. The second step is assayed immunochemically.

HDL, HDL_2 and HDL_3 were active in both assays. ApoHDL had no esterase activity but retained a portion of the immunochemical activity. The esterase activity was partially restored by recombination of apoHDL with PC alone and completely restored by PC + CE. Either R-thr or R-gln could substitute for apoHDL in these assays. Tangier plasma had very low levels of endotoxin-inactivating activity. Plasma from patients with abetalipoproteinemia or Tangier plasma supplemented with normal HDL had normal activity.

6. Quaternary Structure of HDL: EM and molecular weight (MW) studies from other laboratories suggest that HDL is composed of 30,000 MW subunits. Whether these subunits are dimers of R-thr (16,000 MW) or R-gln (14,000 MW) or whether they are mixed dimers is unknown. In addition, it is not known whether all HDL molecules have the same protein composition. Because of the importance of such information in understanding the structure of the intact HDL macromolecule, studies were begun to determine the relationship of R-thr to R-gln in HDL.

R-thr and R-gln were covalently bound to Sepharose 4B and used to prepare purified antiR-thr and antiR-gln from antiHDL. In preliminary experiments, antiR-thr precipitated 100% of HDL molecules, antiR-gln only 45-55%. The HDL remaining in solution after antiR-gln precipitation contained only R-thr. Hence, there are at least two forms of HDL, one containing only R-thr and one containing approximately equimolar quantitites of R-thr and R-gln. Polyacrylamide gel electrophoresis of HDL yielded two lipid-containing bands. Protein analysis of these bands has not been completed.

7. Composition of HDL from Tangier Heterozygotes: As reported last year, the trace amount of HDL present in homozygous patients with Tangier disease has an abnormal composition (R-thr/R-gln = 1/12; normal = 3/1). However, no immunochemical or electrophoretic difference exists between the isolated Tangier apoproteins and their normal counterparts, suggesting the disorder may be inadequate synthesis of an otherwise normal R-thr. A logical extension of this hypothesis is that Tangier heterozygotes should have a partial defect in R-thr synthesis. Preliminary experiments confirm this. The composition of HDL from two Tangier heterozygotes was abnormal (R-thr/gln = 1.5/1, normal = 3/1).

Significance to Biomedical Research and the Program of the Institute:

Knowledge of the basic structure of the plasma lipoproteins is vital to our understanding of normal and abnormal lipid transport. This is specifically germane to vascular disorders associated with abnormal plasma lipid concentrations.

Proposed Course:

1. Primary sequence studies of R-gln and, in particular, of lipid binding peptides isolated following partial degradation of R-gln will be continued. The lipid-binding properties of these peptides will be explored in detail. In addition, lipid derivatives will be prepared which will covalently label the lipid-binding site to aid in its identification and characterization.

2. Studies of the effect of relipidation on the structure of R-thr and R-gln will be continued. In particular the effect of relipidation on fluorescence properties of the apoproteins will be explored. New methods of relipidation using insolubilized LCAT instead of sonification are being developed which should provide a reconstituted HDL more nearly resembling native HDL than is currently possible. Additional studies of the "disk-sphere" transition seen in the electron microscope will be undertaken using differential scanning calorimetry to moniter the physical state of the lipids

in each conformation.

3. Ongoing studies of the quaternary structure of HDL will be completed. The two forms of HDL will be isolated and characterized by preparative polyacrylamide gel electrophoresis. Columns of Sepharose-antiR-thr, Sepharose-antiR-gln and parachloromercuribenzoate-Sepharose have been prepared for use in separating HDL molecules based on their content of R-thr and R-gln. Dimethylsuberimidate has been synthesized for use in crosslinking neighboring subunits.

4. Investigations of the protein defect in Tangier disease will be continued. A radioimmunoassay of R-thr and R-gln is being developed which will allow us to extend the current studies on Tangier heterozygotes as well as detect aberrations of R-thr and R-gln metabolism in other disease states. The lipid and protein composition, molecular size and electron microscopic appearance of Tangier VLDL, LDL and HDL will be investigated. Radioactive turnover studies of normal and Tangier HDL and VLDL are also planned and should provide important information about the pathophysiology of Tangier disease, the metabolism of HDL and its role in VLDL metabolism.

Publications:

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Abstracts:

1. Lux, S.E., R. I. Levy, A.M.Gotto, and D.S.Frdrickson. Studies of the protein defect in Tangier disease. Pediat.Res. 4: 439-440, 1970.

2. Lux, S.E., R. Hirz, R.I.Shrager and A.M.Gotto. Lipid induced structural changes in human plasma high density lipoproteins. Biophysical J. 11: 1812, 1971.

3. Hirz,R., S.E.Lux, and A.M.Gotto. Lipid-protein interactions in native and reconstituted high density lipoproteins studied with a spin label. Circulation 42, Supplement III:- III-8, 1970.

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Serial No. NHLI-284(c)
1. Molecular Disease Branch
2. Section on Molecular Disease
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Glycolipids and Other Lipid Constituents of Normal Human Liver
Previous Serial Number:	NHI-127(c)
Principal Investigators:	Howard R. Sloan, M. D.,Ph.D. Donald S. Fredrickson, M. D.
Other Investigators:	Seniye Temel, B. S. Barbara Davis, B. S. Stephen Demosky, B. S.
Cooperating Units:	None

Project Description:

Objectives: The principal objective of this study is development of an inclusive method for determination of simple and complex lipids, including the neutral glycolipids and gangliosides in plasma and tissues, for purposes of improved diagnosis of the lipidoses. The human liver has been used as the prototype tissue source. The methodology is particularly needed to gain insight into the biochemical interrelationships of the neutral glycolipids to each other and to other lipid classes.

Methods: Lipids were extracted from liver specimens (0.5 - 1.0 gms wet weight) with C:M (2:1) and partitioned according to Folch. The lower phase was subjected to silicic acid chromatography. The following were obtained: (1) neutral lipids, (2) neutral glycolipids (contaminated with small amount of phospholipid), and (3) remaining phospholipids. Cholesterol and glycerides were determined by automated techniques. Phospholipids in the glycolipid fraction were removed by alkaline methanolysis. The glycolipids were separated by thin-layer chromatography and their carbohydrate composition determined by gas chromatography of trimethylsilyl (TMS) derivatives following acid methanolysis. This technique also permitted the determination of the amount of each glycolipid present in the liver sample. The phospholipid composition was determined on a separate aliquot of the lower phase. The gangliosides in the upper phase were studied by thin-layer and gas-liquid chromatography, and by the determination of the amounts of sialic acid in each class of gangliosides.

Major Findings: An analysis of the lipids in normal human liver was developed that is particularly designed to assist in the classification and study of lipid storage diseases. Special emphasis has been given to

determine the quantity and composition of the neutral glycolipid classes and predominant ganglioside (G_{M3}). The neutral glycolipid content of 0.19 (S.D. $\pm$ 0.11) µmoles per gm wet tissue represented 0.4 percent of the total lipid in liver. Ceramide dihexoside was the most abundant neutral glycolipid. The mean contents of cholesterol, glycerides, and total phospholipids were 3.9, 19.5, and 25.1 mg/gm wet weight, respectively. The relative amounts of seven different phospholipid classes were also determined; these included cardiolipin (diphosphatidyl glycerol) which constituted 3.9 percent of the liver phospholipids. The normal amounts of each of six ganglioside classes has also been determined.

Significance to Biomedical Research and the Program of the Institute: Tissue storage of lipids is a principal process in atheromata formation. Studies which elucidate abnormalities in lipid metabolism and provide new means for the detection of biochemical defects offer useful new approaches which may yield specific clues to the process of atherogenesis.

Proposed Course:

1. Investigation of abnormal human livers and particular emphasis on patients with previously "unclassified" lipidoses.

Publications:

Kwiterovich, P. O., Sloan, H. R., and Fredrickson, D. S.: Glycolipids and other lipid constituents of normal human liver. J. Lipid Res., 11,322, 1970.

Serial No. NHLI-285(c)
1. Molecular Disease Branch
2. Section on Molecular Disease
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:	Tissue Lipidoses: Abnormal biochemistry in tissue lipid storage diseases.
Previous Serial Number:	NHI-132(c)
Principal Investigators:	Howard R. Sloan, M. D., Ph.D. Donald S. Fredrickson, M. D. Jan Breslow, M. D.
Other Investigators:	Seniye Temel, B. S. Barbara Davis, B. S. Stephen Demosky, B. S. Briston Williamson
Cooperating Units:	B. William Uhlendorf, Ph. D. Laboratory of Viral Immunology, DBS; Carl Hansen, Ph. D., DRS, Laboratory Aids Branch; Victor J. Ferrans, M. D., Ph. D., Cardiology Branch, NHLI

Project Description:

Objectives: To improve the knowledge of the biochemical basis of the genetically determined tissue lipid storage diseases as well as to improve diagnostic techniques for both the patients and possible heterozygous carriers. Preliminary findings in this laboratory several years ago indicated that cells derived from the bone marrow of patients with Niemann-Pick disease had an abnormal sphingomyelin content. The study of the accumulation of sphingomyelin in tissue culture cells has now been extended to studies of the enzymatic defects in several lipid storage disorders. Specifically, it has now been demonstrated that bone marrow and skin fibroblasts derived from patients with Niemann-Pick disease, Types A and B, have a markedly depressed level of the enzyme which cleaves sphingomyelin. This type of investigation has been extended to studies of sulfatide lipidosis, Gaucher's disease, Fabry's disease, and generalized gangliosidosis. These diseases offer a model of great interest for study of the lipid storage diseases.

Methods: Patients with Niemann-Pick disease, generalized gangliosidosis, and patients with other lipid storage disorders are admitted for evaluation, including biopsy for chemical studies if indicated. In addition, bone marrow and skin biopsies are obtained and the resulting tissue is propagated in tissue culture. The cells are cultivated and processed as described in previous reports. Homogenates and sonicates of solid tissues and tissue culture cells are prepared for enzymatic and lipid composition studies as described previously.

Methods have been developed for labeling five complex lipids with radioactive isotopes. Sphingomyelin, galactosyl ceramide, glucosyl ceramide, ganglioside G_{M1}, and sulfatide have been labeled specifically in the choline, galactose, glucose, galactose, and sulfate moiety. With the use of these substrates and several commercially available nitrophenyl derivatives, tissue culture cells from over 140 individuals have been studied.

In addition, tissue culture cells have been grown on cover slips and have been incubated with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside gal (BCI). Peripheral leukocyte cultures and 10μ thick sections of human liver have also been incubated with this histochemical substrate. The blue color, resulting from the formation of indigo following cleavage of the terminal galactose of this molecule indicates the presence of β-galactosidase in the tissue culture cells. The method has recently been extended to BCI-glucose and BCI-sulfate.

A novel method of purifying sphingolipid hydrolases has also been developed. Sphingomyelin has been deacylated and the resulting sphingosylphosphorylcholine has been covalently bonded to sepharose with water soluble carbodiimides. Sphingomyelin acid hydrolase binds avidly to the altered sepharose. The same technique has been employed with glucosyl ceramide and sulfatide.

Major Findings:

1. A marked decrease in sphingomyelin cleaving enzyme was demonstrated in tissue cultures from patients with the classical infantile form of Niemann-Pick disease and the patients with the juvenile-visceral form of this disorder. The enzymatic activity is normal in both tissue and tissue cultures of patients with types C and D Niemann-Pick disease.

2. Fibroblasts derived from skin and bone marrow of three patients with generalized gangliosidosis have a markedly depressed activity of β-galactosidase as demonstrated with both orthonitrophenylgalactopyranoside and ganglioside G_{M1}-^{3}H.

3. Fibroblasts derived from the skin of six patients with metachromatic leukodystrophy (sulfatide lipidosis) have been studied. These cells contain a markedly decreased activity of sulfatidase activity employing ceramide galactose-3-sulphate-S35 as substrate. With para nitrocatechol sulfate as substrate the preparations from patients with sulfatide lipidosis have markedly depressed arylsulfatase A activity but normal arylsulfatase B activity.

4. The activity of four enzymes: sphingomyelin cleaving enzyme, glucosyl ceramide cleaving enzyme, G_{M1}-β- galactosidase, and ceramide galactose-3-sulfate sulfatase, have been determined in several fibroblast lines derived from human amniotic fluid. Fibroblasts derived from amniotic fluid obtained as early as the third month of gestation have measurable levels of the various specific hydrolyases. This finding might permit the intrauterine diagnosis of the three disorders characterized by absence of these enzymes as early as the 22nd week of gestation.

5. Fibroblasts derived from the amniotic fluid have also been incubated with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside. These fibroblasts have readily demonstrated activity toward this substrate. This finding may permit the intrauterine diagnosis of G_{M1} gangliosidosis within two days of obtaining amniotic fluid at amniocentesis. Two pregnancies at risk for G_{M1} gangliosidosis have been monitored successfully by this method. The infants, as predicted, are free from this disorder.

6. The fm mouse strain has been well established now at NIH. These mice have a lipidosis which seems somewhat similar to adult of Type C human Niemann-Pick disease. The sphingomyelin and cholesterol content in the thymus is significantly higher in affected animals than in normals. The content of sphingomyelin cleaving enzyme in the liver, thymus, and spleen of affected mice, however, was not depressed when compared with control mice of the same strain. Further studies have been performed which demonstrate unequivocally that the major compound stored in sphingomyelin and not lysolecithin as previously reported. In addition, marked accumulations of glycolipids and gangliosides have been demonstrated in the affected mice. No specific ganglioside or glycolipid class is accumulated.

7. Sphingomyelin cleaving enzyme from normal human liver has been purified by a combination of the techniques of classical enzyme biochemistry and affinity chromatography employing sphingosylphosphorylcholinesepharose. A pre paration has been obtained in which the sphingomyelin-cleaving enzyme has been purified over 10,000-fold. Polyacrylamide gel electrophoresis reveals the presence of only one major and two minor protein components.

8. Sulfatide-sulfatase from normal urine has been extensively purified by a combination of classical and affinity chromotographic techniques. Polyacrylamide gel electrophoresis reveals that sulfatide-sulfatase constitutes more than 95% of the protein present.

9. Glucosyl ceramide from normal liver has also been extensively purified by these techniques. β-glucosidase comprises more than 90% of the protein in the purest preparation.

10. The diagnostic capabilities of the Molecular Disease Branch have been expanded to include all of the major lipid classes and all of the carbohydrates important in mammalian biochemistry.

Significance to Biomedical Research and the Program of the Institute:
Tissue storage of lipids is a principal process in atheroma formation. Studies which elucidate abnormalities in lipid metabolism and which provide new means for the differentiation of biochemical defects offer useful new approaches which may yield specific clues to the process of atherogenesis. Moreover, a perpetuation of metabolic disorders in tissue culture cells makes possible a detailed approach to the pathogenesis and control of metabolic disorders. In addition, this perpetuation may facilitate the study of the genetic defect in the various lipid storage disorders.

Proposed Course:

1. Quantification of the sphingomyelin cleaving, the β-glucosidase, the G_{M1}-β-galactosidase, an d the sulfatide sulfatase enzyme activities in parents (heterozygotes) of patients in comparison with normals.

2. The mechanism of at least four new previously undescribed lipidoses is now under study.

3. Continued study of the fm strain of mice. Attempts will be made to study the rate of synthesis of sphingomyelin and cholesterol in young animals before the disease becomes clinically manifest. The nature of the glycolipid accumulation will also be investigated.

4. Determination of the defect in the primary protein structures of the abnormal sphingomyelin cleaving enzyme in two types of Niemann-Pick disease.

5. Determination of the defect in the primary protein structures of the abnormal sulfatide sulfatase in sulfatide lipidosis and of the abnormal β-glucosidase in Gaucher's disease.

6. Further studies of these enzymatic activities in tissue culture cells derived from amniotic fluid.

Publications:

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Serial No. NHLI-286

1. Molecular Disease and Clinical Endocrinology Branches
2. Section on Peptide Chemistry
3. Bethesda, Maryland 20014

PHS-NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title:

1. Chemistry and physical properties of Bovine Parathyroid Hormone (PTH):
   (a) isolation and characterization of bovine PTH
   (b) complete amino acid sequence of bovine PTH
   (c) physiochemical properties of bovine PTH
2. Chemistry and immunological properties of Thyrocalcitonin (TC):
   (a) comparison of the physical chemical properties of procine, bovine, human and salmon TC
   (b) characterization of the amino acid residues involved in the immunological reactivity of porcine TC
3. Chemistry and conformation of the carboxyl terminal alanine peptide (apoVLDL-ala) derived from the very low density lipoprotein particle
   (a) primary structural studies of apoVLDL-ala
   (b) physiochemical properties of apoVLDL-ala

Previous Serial No.: NHLI-309

Principal Investigators: H. Bryan Brewer, Jr., M.D.
Richard Shulman, M.D.

Other Investigators: Rosemary Ronan, B.A.
Katherine Wehrly, B.A.

Cooperating Units: Paul Munson, Ph.D., Department of Pharmacology , University of North Carolina; Robert Schlueter, Ph.D., Armour Pharmaceutical Company; Harold Edelhoch, Ph.D., National Institute of Arthritis and Metabolic Diseases, Clinical Endocrinology Branch; Peter Herbert, M.D. and Robert I. Levy, M.D., Section on Lipoproteins, Molecular Disease Branch, NHLI.

Project Description:

(1a) Objective: Isolation and characterization of bovine parathyroid hormone.

Methods Employed: Parathyroid hormone was isolated in homogeneous form from a trichloroacetic acid extract of bovine parathyroid glands. The purification of scheme involved fractionation of the partially purified hormone by gel filtration followed by ion exchange chromatography on CM-sephadex.

Major Findings: The purified bovine hormone was shown to be homogeneous by disc gel electrophoresis, thin layer chromatography, and Edman amino terminal analysis. The isolated hormone contains 84 amino acids and has a biological activity of approximately 3000 USP units/mg. The amino acid composition of the isolated hormone was determined by a combination of acid hydrolysis and total enzymatic digestion and is as follows: Lys_9, His_4, Arg_5, Trp_1, Tyr_1, Phe_2, Leu_8, $ileu_3$, Met_2, Val_8, Ala_7, Pro_2, Gly_4, Glu_6, Gln_5, Ser_8, Asp_6, Asn_3.

(1b) Objective: Determination of the complete covalent structure of bovine parathyroid hormone.

Methods Employed: The complete amino acid sequence of PTH was determined by the isolation and characterization of peptides derived from enzymatic and chemical cleavages at specific amino acid residues, and sequential Edman degradations on the intact molecule and tryptic fragments.

Major Findings: The individual peptides obtained from the various methods of cleavage were isolated in homogeneous form by gel filtration, ion exchange chromatography, or preparative thin layer chromatography. The combined results provided a series of overlapping peptides for the entire molecule, as well as the definitive position of several residues in

the sequence. The complete amino acid sequence of bovine PTH was determined by sequential Edman degradations utilizing the manual Edman technique and the automated Beckman sequencer. Three degradations were performed on the intact hormone with the sequencer resulting in the definitive sequence of 66 of the 84 residues. The remainder of the sequence was determined by manual degradations on the isolated tryptic peptides. The combined results provided a unique amino acid sequence for the hormone which is as follows: Ala-Val-Ser-Glu-ileu-Gln-Phe-Met-His-Asn-Leu-Gly-Lys-His-Leu-Ser-Ser-Met-Glu-Arg-Val-Glu-Trp-Leu-Arg-Lys-Lys-Leu-Gln-Asp-Val-His-Asn-Phe-Val-Ala-Leu-Gly-Ala-Ser-ileu-Ala-Tyr-Arg-Asp-Gly-Ser-Ser-Gln-Arg-Pro-Arg-Lys-Lys-Glu-Asp-Asn-Val-Leu-Val-Glu-Ser-His-Gln-Lys-Ser-Leu-Gly-Gln-Ala-Asp-Lys-Ala-Asp-Val-Asp-Val-Leu-ileu-Lys-Ala-Lys-Pro-Gln.

(1c) Objective: To evaluate the degree of secondary and tertiary structure of bovine PTH.

Methods Employed: The degree of secondary structure (α-helix or β structure) of PTH in aqueous solution was determined by circular dichroism (CD), optical rotatory dispersion, and infrared spectroscopy. Tertiary interactions of the hormone were assessed by the fluorescence and near ultraviolet circular dichroism of the aromatic amino acids. Studies were performed on the native hormone, and the hormone in 6M guanidine and 90% 2-chloroethanol.

Major Findings:

Secondary Structure: The major circular dichroic activity of PTH was near 200 mu, the region of optical activity of the random form of the peptide bond. The optical activity at 222 mu, the $n \rightarrow \pi^*$ transition of the α helix, suggests that PTH contains 10-15% α helical structure. In 6M guanidine, the spectrum resembled a random coil; in 2-chloroethanol PTH formed a structure containing 50% helix.

Tertiary Structure: The fluorescence emission spectrum of PTH revealed a minor band at 302 mu, and a major band at 348 mu, corresponding to the single tyrosine and tryptophan residues respectively. No change in the spectrum was observed in 6M guanidine indicating that the chromophores were solvent accessible. On alkaline titration a 35% loss of tryptophanyl fluorescence was observed, which was normalized in 6M guanidine. These results indicate that there is a conformational association between the tyrosine and tryptophan residues which can be normalized with a denaturing solvent.

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The near UVCD spectrum of PTH in aqueous solution revealed minor bands which could be assigned to the phenylalanine (s), tyrosine, and tryptophan residues.

In summary, it appears that bovine PTH exists predominately in an unordered conformation in aqueous solution with 1-2 turns of helix. A structural association can be demonstrated between the tyrosine and tryptophan. PTH does not possess a rigid conformation and a coil ⇌ helix equilibrium may exist in aqueous solution with guanidine shifting the equilibrium in favor of the coil and 2-chloroethanol in favor of the helix. The latter type of transition may occur at the membrane receptor site.

(2a) Objective: Salmon TC (STC) is 10-15 fold more biologically active than the TC hormone obtained from the human (HTC), porcine (PTC), and bovine (BTC) species. The objective of this study was to determine the conformation of the salmon hormone and to compare its physical properties with those of other species, thereby gaining further insight into the structure and function of the thyrocalcitonin hormone.

Methods Employed: Analysis of the secondary and tertiary structure of the salmon TC were similar to those employed for PTH (section 1(c)).

Major Findings:

Secondary Structure: The optical activity at the $n \rightarrow \pi^*$ transition of the α helix indicates that the salmon hormone is predominately a random coil with 1-2 turns of helix in aqueous solution. In 6M guanidine the spectrum resembled that of a random coil, while in 2-chloroethanol STC formed a structure containing 25-30% helix. The helical content in aqueous solution is therefore similar to the previous results we have obtained with porcine, bovine, and human TC. The helical content of STC in 2-chloroethanol is similar to HTC (25% helix), and approximately half that of PTC and BTC (50% helix). This result may reflect the substitution at position 23 (STC, HTC) of a proline residue in place of serine (PTC, BTC) which would tend to inhibit helix formation in non-aqueous solvents.

Tertiary Structure: The fluorescence spectrum of STC revealed a peak at 302 mu, similar to free tyrosine in aqueous solution. The pK of the tyrosine determined by fluorometric and spectrophotometric titration was normal indication complete solvent accessibility of the hydroxyl group of the tyrosine residue. The near UVCD of STC revealed a peak at 275 mu which could be assigned to the single tyrosine residue indication that this residue had restricted rotational freedom.

These combined results indicate the STC exists predominately as a random coil in aqueous solution and is therefore similar to BTC, PTC, and HTC. It appears, therefore, that the increased biological activity of STC is a function of the primary amino acid sequence of the molecule rather than a unique conformation.

(2b) Objective: To determine the amino acid regions of the porcine TC molecule which are responsible for the immunological reactivity of the hormone with the porcine TC antibody.

Methods Employed: Selected amino acid regions of the porcine TC molecule were analyzed by the utilization of synthetic peptide fragments, as well as peptide derived from tryptic digestion of the native hormone.

Major Findings: Three peptides were isolated in homogeneous form following tryptic digestion of native porcine TC. These fragments consisted of residues 1 to 14, 15 to 21, and 22 to 32. In addition, the synthetic fragments of residues 1 to 9, 10 to 32 were obtained from Ciba Pharmaceutical Co. (Basle, Switzerland). There was no immunological cross reactivity of the three tryptic peptides with the native hormone in the immunoassay system. The synthetic amino terminal peptide (residues 1 to 9) was also immunologically unreactive; the synthetic fragment residues 10 to 32, however, retained a high degree of immunological activity. These studies indicate that the middle and carboxyl terminal regions of the hormone contain the residues required for immunological activity with this particular antiserum. Further cleavage of this region, as was obtained with tryptic digestion, destroyed the reactivity. These studies are consistent with our previous studies in which reduction and carboxymethylation of the amino terminal disulfide resulted in no loss of immunochemical reactivity. In addition, there was poor immunological cross reactivity of the porcine antibody with human and salmon TC. The latter two species have an amino terminal sequence which is similar to the porcine species, however, the middle and carboxyl terminal region are significantly different.

In summary, it appears that the residues required for immunological activity are located in a 21 amino acid peptide located in the middle and carboxyl region of the molecule. As yet, no smaller fragment has been obtained which retains reactivity.

(3a) Objective: To obtain primary structural information on apoVLDL-ala, thereby gaining insight into the structural basis for lipid binding and immunochemical reactivity of the plasma lipoproteins.

Methods Employed: Peptide fragments of apoVLDL-ala were prepared by cleavage of the intact peptide by enzymatic and chemical techniques. Sequential degradations of the intact molecule and peptide fragments were

performed by the manual and automated Edman procedure.

Major Findings: A number of peptide fragments derived from trypsin and cynogen bromide cleavage have been isolated and characterized. A number of these peptides and the intact polypeptide have been further analyzed by the Edman procedure. These studies have enabled the construction of a preliminary structural model for apoVLDL-ala.

(3b) Objective: Evaluation of the secondary and tertiary structure of apoVLDL-ala.

Methods Employed: Similar to those employed for PTH (section 1c).

Major Findings: The optical activity at the $n \rightarrow \pi^*$ transition of the α helix suggests that apoVLDL-ala contains 10-15% helix. The spectrum in 6M guanidine is similar to that of randomly coiled polypeptides. In 2-chloroethanol apoVLDL-ala assumed a structure containing approximately 80-90% helix. The fluorescence spectrum of apoVLDL-ala revealed a peak at 348 mu, characteristic of free tryptophan in aqueous solution. A 30% loss of tryptophanyl fluorescence was observed during alkaline titration. An identical curve was obtained in 6M guanidine indicating a close proximity of a tyrosine (s) and tryptophan (s) in the primary amino acid sequence. Spectrophotometric titration of the tyrosine revealed a pK near 10, similar to free tyrosine in aqueous solution. These studies indicate that apoVLDL-ala exists predominately as a random coil in aqueous solution. The helical type of transition, however, may occur when apoVLDL-ala interacts with its normal complement of lipid.

Significance to Biomedical Research and the Program of the Institute: This work is directed toward a greater understanding of the structure, function, and physiological role of polypeptides in cellular metabolism. Determination of the complete covalent structure of the parathyroid hormone will now enable more detailed studies on the chemistry and physiology of the hormone, as well as the synthesis of chemical analogs which should further increase the understanding of calcium metabolism and metabolic bone disease. Knowledge of the primary structure and physical chemical properties of the plasma lipoproteins is essential to an understanding of the amino acid residues responsible for the immunological reactivity and lipid binding of the plasma lipoproteins. This is particularly germane to the elucidation of the pathogenesis and mechanism of abnormal lipid transport in atherosclerosis.

Proposed Course:

1. It is planned during the next year to complete the covalent

structure of apoVLDL-ala. In addition, a systematic investigation will be undertaken to determine the amino acid residues responsible for the immunological reactivity and lipid binding properties of apoVLDL-ala. These studies will involve lipid-peptide recombination experiments, which will be assessed by spectral techniques on the native polypeptide, as well as peptide fragments derived from enzymatic and chemical cleavages of the intact molecule.

2. The systematic study of the amino acid regions of thyrocalcitonin and parathyroid hormones that are important for biological and immunological activity will be continued by preparing derivatives through limited cleavage of the molecule or by selective modification of individual amino acid residues with chemical reagents. The studies on the conformation of the hormones will be extended to include analysis of the effects of iodination and modification of single amino acid residues in the secondary and tertiary structure of the hormones. A series of studies will be undertaken to approximate the hormone-membrane interaction at the cellular receptor site by experiments dealing with hormone-lipid, lipoprotein recombination. It is hoped that these studies on intact hormones and peptide fragments will give further insight into the amino acid residues or types of residues (hydrophobic, hydrophilic, charged, etc.) in polypeptides which are responsible for lipid binding.

Publications:

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Pisano, J. P., Bronzert, T., and Brewer, H. B., Jr.: Advances in the gas chromatographic analysis of amino acid phenyl- and methyl throhydantins. Analytical Biochemistry (in press).

Fales, H. M., Nagai, Y., Milne, G. W. A., Brewer, H. B., Jr., Bronzert, T. J., and Pisano, J. P.: The use of chemical ionization mass spectrometry in the analysis of the amino acid phenylthrohydantins derivatives formed during the Edman degradation of proteins. Analytical Biochemistry (in press).

Serial No. NHLI- 287
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Sterol Synthesis in Mammalian Arteries.

Previous Serial No.: None.

Principal Investigator: Joel Avigan, Ph.D.

Other Investigators: Carolyn D. Williams, Ph.D.
Sam J. Bhathena, Ph.D.
Marta E. Schreiner, B.S.

Project Description:

Objectives: To study factors affecting the rate of synthesis of sterols and other cellular components in arterial tissue.

Methods: Rabbit aortic tissue, consisting of intima and media, was prepared, either in the form of whole strips or of a mince, and incubated aseptically in medium containing Krebs-Ringer bicarbonate buffer, glucose and either whole or solvent-extracted serum. The tissue was subsequently washed, incubated with radio-labeled acetate, thymidine or alanine, and radioactivity of sterols, fatty acids and DNA or proteins was determined.

Major Findings: Synthesis of nonsaponifiable lipids and of fatty acids from labeled acetate in minced or otherwise damaged arterial tissue is greatly increased (by a factor of 20 or larger) after incubation for 16 hours or longer with solvent-extracted serum. This finding is similar to what was previously observed in fibroblasts and leukocytes (1970 Reports Nos. NHLI-317 and 318). Protein and DNA synthesis was also greatly increased. Whole strips of aorta incorporate considerably more acetate into lipids than does minced tissue, but in the strips lipid synthesis from acetate was also increased several-fold following incubation with solvent-extracted serum. Incorporation of labeled glucose and of ^3H_2O into lipids followed a similar pattern, demonstrating that the above results reflect genuine changes in synthetic activities and are not due to artifacts produced by nonphysiologic precursors. In the aorta, in which acetate incorporation was increased by in vitro preincubation, a larger part of the radioactivity of nonsaponifiable lipids was in cholesterol, compared with the nonstimulated control. In contrast to previous findings with fibroblasts and leukocytes, where lipid synthesis was strongly inhibited by incubation with whole serum, lipid synthesis in aorta was stimulated after overnight exposure to a serum-containing medium, although to a lesser extent than with a solvent-extracted serum. These results indicate that the feedback inhibition of sterol synthesis in vitro is less effective in arterial tissue

than it is in fibroblasts and leukocytes, while activation of the biosynthetic processes by serum proteins occurs with both types of preparations.

Significance to Heart Research: The study may contribute to heart research in two ways: (1) By determining conditions under which sterol synthesis in arteries is increased and by examining the role of sterol synthesized in situ in the process of atherogenesis, and (2) by studying the effect of experimental injury on lipid synthesis and other biosynthetic processes correlated with cell growth and proliferation, the latter being an important factor in atherogenesis.

Proposed Course: The nature of the observed stimulation of lipid synthesis and of other synthetic reactions is to be further investigated. The depression in the above activities following damage and the puzzling reversal thereof in vitro should also be studied, with the aim of forming an experimental model for the biochemical response to arterial injury.

Publications: None.

Serial No. NHLI-288
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Cholesterol Synthesis in Mammalian Cells in vitro.

Previous Serial No. NHLI-317

Principal Investigator: Joel Avigan, Ph.D.

Other Investigators: Carolyn D. Williams, Ph.D.

Project Description:

Objectives: To study the effects of serum proteins and lipids on cholesterol synthesis in mammalian cells in vitro.

Methods: The previous studies in skin fibroblast cultures were expanded to include freshly isolated human blood leukocytes. The cells were obtained either in a form of a suspension or monolayers formed on glass surface. The cells were incubated for periods of one day or longer with media containing either whole serum or a solvent-extracted serum. The cells were subsequently washed and the rate of biosynthesis of lipids and proteins assayed by incubation in a protein-free medium with labeled acetate or other radioactive precursors. The products were finally isolated and their radioactivity determined.

Major Findings: In a typical experiment, preincubation of leukocyte monolayers with solvent-extracted serum caused a 9-fold increase of incorporation of labeled acetate into nonsaponifiable lipids over the amount incorporated by freshly isolated cells. There was a lesser stimulation of incorporation of mevalonate into nonsaponifiable lipids and little or no effect on incorporation of acetate into fatty acids or of alanine into proteins. Preincubation with a similar concentration of whole serum was either inhibitory or without effect. Thin-layer chromatography of the radioactive nonsaponifiable lipids isolated from the stimulated cells revealed that 65% of the radioactivity was in the 27-carbon sterol fraction and nearly 80% in the combined squalene and sterol fractions.

Significance to Heart Research: These results, as well as those previously obtained with human fibroblasts, are consistent with an inhibition of cholesterol synthesis by exogenous sterol in the medium. Since freshly prepared leukocytes are cells that have not been adapted to growth in culture, the findings indicate that feedback regulation of cholesterol synthesis in vitro is not limited to cells grown in culture, but characterizes at least

some mammalian cells in their native state. For obvious reasons, leukocytes may turn out to be a convenient tissue for the study of sterol synthesis in individual human subjects.

Proposed Course: The nature of the above described in vitro stimulation of sterol synthesis and of the suppression of synthesis by lipids in the medium should be studied further. The behavior of cells derived from individuals suffering from Type II and Type III hyperlipemias could also be studied and compared with that of cells from normal subjects. It should be indicated that attempts to reveal significant differences between lipid biosynthesis in skin fibroblasts from hyperlipemic individuals and that from normal subjects (1970 Report No. NHLI-318) have hitherto been unsuccessful.

Publications:

Avigan, J., Williams, C.D., and Blass, J.P.: Regulation of sterol synthesis in human skin fibroblast cultures. Biochim. Biophys. Acta, 218: 381-384, 1970.

Serial No. NHLI-289
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Mechanism of Release of Histamine and other Vasoactive Substances from Mast Cells.

Previous Serial No.: NHLI-319

Principal Investigators: James H. Baxter, M.D.
Vincent Manganiello, M.D., Ph.D.
Martha Vaughan, M.D.

Other Investigators: Carlos Schultz

Cooperating Units: Z. Horáková and M. A. Beaven, Ph.D., Experimental Therapeutics Branch, NHLI.

Project Description:

Objectives: To study the effects of selected drugs on the dextran-induced release of histamine from mast cells, in vivo and in vitro, and on the levels of cyclic AMP in mast cells, thereby to identify mechanisms controlling the release of histamine.

Methods:
(1) Rats were injected intravenously with dextran, after pretreatment with various drugs, and the anaphylactoid reaction, in vivo histamine release, and level of blood sugar were evaluated.
(2) Peritoneal mast cells (impure) were treated with dextran, with and without prior addition of various drugs, and the degree of histamine release was determined.
(3) The effect of selected drugs on cyclic AMP levels of peritoneal cells was determined. In some experiments, the peritoneal cells were fractionated by using Ficoll gradients.

Major Findings:

(1) Additional studies of the effects of drugs on the dextran reaction and histamine release in vivo, and of the relation of drug inhibition of the reaction to hyperglycemia gave results which were essentially as described last year (Fed. Proc. 29, 2088, 1970). The dextran-induced release of vasoactive substances from mast cells appeared to be mediated by dextran itself, rather than by a secondary factor generated in the plasma (indicating that dextran binding studies might be relevant).

(2) In vitro studies with peritoneal cells (Fed. Proc. 30, 500, 1971).
(a) Addition of dextran (ca 5 mg/ml) to peritoneal cells caused release of histamine (usually 10-30% of total).

(b) Within an individual experiment, histamine release increased as dextran concentration was increased (0.02 - 7.7 mg/ml).

(c) Histamine release usually plateaued within 5 minutes (even with low dextran), so that rates of release could not be studied. Studies were made of total release after 10-15 minutes, although the mechanistic significance of a change in total release is not entirely clear.

(d) Approximately 50% inhibition of release (i.e., of plateau level) was produced by theophylline (3×10^{-4} M), dibutyryl cyclic AMP (10^{-4}), phenoxybenzamine (5×10^{-5}), nicotinamide (10^{-3}), glucose (4×10^{-3}), ethanol (5×10^{-2}). Higher concentrations gave almost complete inhibition. Maximal inhibition by isoproterenol (up to about 60%) occurred at 10^{-8} M. Epinephrine (10^{-7} M) and norepinephrine (10^{-6} M) were also partially effective. PGE_1 inhibited only at high concentrations (10^{-5} M), and insulin and glucagon did not inhibit. NaF (10^{-2} M) released most of the cellular histamine without requiring dextran. These results are similar to those previously obtained in vivo.

(e) The inhibition of histamine release from mast cells by drugs that increase cyclic AMP in other situations, suggests that the release from mast cells may be regulated by cyclic AMP. However, it is not certain that the inhibition was produced by specific action on cyclic AMP, rather than through some other metabolic effect (see following section on mast cell cAMP).

(3) Studies on cyclic AMP levels of peritoneal cells.
(a) The rat peritoneal cells consisted of about 5% mast cells and 95% macrophages and leukocytes. By mass, however, the mast cells composed about 20-25% of the total; the mast cells contained the major portion of the total histamine.

(b) The mixed peritoneal cells contained considerable quantities (per mg of protein) of cyclic AMP, and the peritoneal fluid (after the cells had been removed) contained a total amount approximately equal to that in the cells. Incubation of the cells with isoproterenol (3×10^{-6} M) plus theophylline (1×10^{-3} M) considerably increased the cyclic AMP of cells plus medium.

(c) After fractionation of the peritoneal cells, the non-mast cells showed a definite increase in cyclic AMP following treatment with theophylline plus isoproterenol. However, the mast cells (although they apparently contained significant amounts of cyclic AMP) have thus far failed to show a similar clear response to the drugs. This failure may have resulted from technical problems, or from the fact that the purified mast cells had largely lost their ability to liberate histamine in response to dextran.

Significance to Heart and Lung Research:
Histamine and other vasoactive substances from mast cells are thought to be involved in the production of allergic and inflammatory reactions.

Proposed Course:

The in vivo study has probably been completed. The response of mast cell cyclic AMP to drugs that inhibit histamine release will be studied further. Additional studies of the loss of responsiveness of mast cells to dextran caused by washing, aging or fractionating of the cells, of binding of dextran to mast cells, and of possible interactions of mast cells and other types of cells will be considered.

Publications: None.

Serial No. NHLI- 290
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Effects of Hormones on Metabolism of Adipose Tissue studied in vitro.

Previous Serial No.: NHLI-323

Principal Investigators: Vincent Manganiello, M.D., Ph.D.
Martha Vaughan, M.D.

Other Investigators: Ferid Murad, M.D., Ph.D.
Sally Stanley, B.S.

Project Description:

Objectives:
To study metabolism of cyclic AMP in fat, particularly as related to hormonal control of lipolysis.

Methods:
Lipolysis measured by enzymatic determination of glycerol; cyclic AMP by activation of phosphorylase and by association with a specific binding protein; adenyl cyclase by the method of Krishna, and protein kinase by phosphorylation of histone with ATP- P^{32}.

Major Findings:

(1) We have completed and published our studies on the effects of cGMP on glycerol production, cAMP levels and cAMP phosphodiesterase activity.

(2) Upon additions of hormones such as epinephrine or ACTH to fat cells, cAMP levels immediately rise, reach a maximal value within 4-7.5 minutes, and then fall despite the continued presence of the hormone. Once cAMP levels fall, further addition of the same hormone or combinations of hormones does not restimulate an increase in cAMP. From experiments with appropriate lipolytic inhibitors, i.e. propranolol and insulin, and by addition of theophylline to fat cells at different times after the addition of hormone, it appears that despite falling cAMP levels, hormone-sensitive adenyl cyclase remains activated. Whether the fall in cAMP levels reflects a decrease in the extent of activation of adenyl cyclase or an increase in the activity of a cAMP phosphodiesterase is not known at present.

(3) We have completed studies begun last year which indicate that the maximal stimulation of fat cell lipolysis by glucagon (relative to that produced

by epinephrine and ACTH) declines markedly with increasing size of donor rats. In addition, relative to epinephrine, the glucagon stimulation of adenyl cyclase is significantly lower in particulate preparations from 400-450 gram rats than from 125-150 gram rats. Such data suggest that a selective loss of the fat cell glucagon receptors occurs during growth of the rat. No similar change in the response of heart adenyl cyclase to glucagon with age was demonstrable.

Significance to Heart Research:
Studies on the mechanism of hormone action, especially epinephrine and glucagon, may have importance and relevance to the mechanisms of their actions on the cardiovascular systems.

Proposed Course:
Study of control of cAMP levels in fat cells will be continued with particular emphasis on the action of insulin and other compounds that are anti-lipolytic.

Publications:

Manganiello, V., Murad, F., and Vaughan, M.: Effects of lipolytic and anti-lipolytic agents on cyclic 3',5'-adenosine monophosphate in fat cells. J. Biol. Chem., 246: 2195-2202, 1971.

Serial No. NHLI- 291
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Formation, Metabolism and Action of Cyclic AMP and Cyclic GMP.

Previous Serial No.: NHLI-325

Principal Investigators: Vincent Manganiello, M.D., Ph.D.
Martha Vaughan, M.D.
Ferid Murad, M.D., Ph.D.

Other Investigators: Robert J. Mason, M.D.
Thomas P. Stossel, M.D.
Sally Stanley, B.S.
Betty Hom, B.S.

Collaborating Units: Warren Evans, M.D.,
Laboratory of Biochemistry, NCI.

Project Description:

Objectives:
To study factors regulating control of cyclic nucleotide metabolism.

Methods and Major Findings:

(1) Cyclic GMP Metabolism: We have developed a simple method employing ion exchange chromatography on DEAE and Dowex-1 resins to study the enzymatic formation of cyclic GMP in vitro, and have also devised a protein binding assay for cyclic GMP similar to that described by Gilman, in order to measure tissue levels of cyclic GMP.

Fat cells hydrolyze cGMP and cAMP at comparable rates. Theophylline inhibits the hydrolysis of both nucleotides. Cyclic AMP inhibits the hydrolysis of cGMP, and cGMP that of cAMP. The pH optima for degradation of cAMP and cGMP are different optima. In frozen-thawed fat cell extracts, although 30-40% of the "low Km" ($2\text{-}4 \times 10^{-6}$ M) enzymatic activity which hydrolyzes cAMP is found in a sedimentable fraction (100,000 x g for 15 minutes), very little low Km activity for cGMP is found. Most of the enzymatic activity which hydrolyzes cGMP in fat cell extracts is soluble.

(2) Cyclic AMP Levels in vitro: Using the binding assay as developed by Gilman, we have examined cAMP levels in several types of cells, including mammalian cells grown in culture, phagocyties of several species, and rat peritoneal cells.

(a) Tissue culture: Contrary to earlier reports, we have found that in hepatoma cells that demonstrate a steroid-induced increase in tyrosine amino transferase, epinephrine increases cAMP levels 2-3 fold. This stimulatory effect requires the presence of theophylline and appears to be inhibited by propranolol but not insulin. Glucagon and PGE_1 do not affect cAMP levels in these cells. Incubation of cells with dexamethasome for 24 hours or 3 days does not induce responsiveness to glucagon. In contrast to hepatoma cells, mouse fibroblasts (L-cells) show only a small cAMP increase in the presence of epinephrine, but a 10-20 fold elevation in the presence of PGE_1. In these cells, theophylline seems to have only a small effect.

(b) Human white blood cells: During phagocytosis of latex beads by guinea pig peritoneal leukocytes and rabbit alveolar macrophages, cAMP levels do not rise demonstrably, whereas human white blood cells, prepared by dextran sedimentation, show a marked prompt increase in cAMP levels. Since the former two-cell types are relatively homogeneous, human WBC were purified by the method of Evans. In these preparations (85-95% pure PMN), cAMP levels rose only slightly during phagocytosis as compared to a mixture of all other WBC types. Such studies suggest either that other types of cells, including platelets, increase cAMP levels in the presence of polystyrene latex beads, that PMN's require the presence of other cell types during phagocytosis to demonstrate the increase in cAMP, or that cAMP levels do not increase in leukocytes during phagocytosis.

Significance to Heart Research:
Studies on cAMP and cGMP metabolism may elucidate the nature of cellular regulatory processes whereby hormones exert their actions on the cardiovascular system.

Proposed Course:
We plan to continue the study of metabolism of cGMP and cAMP in several tissues.

Publications:

Murad, F., Manganiello, V., and Vaughan, M.: A simple, sensitive protein binding assay for guanosine 3',5'-monophosphate.
Proc. Nat. Acad. Sci., 68: 736-739, 1971.

Serial No. NHLI-292
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolism of Lung Tissue and Phagocytic Cells:
(1) Synthesis if dipalmitoyl lecithin by lung and alveolar macrophages,
(2) Characterization of isolated phagocytic vesicles from guinea pig polymorphonuclear leukocytes and rabbit alveolar macrophages,
(3) Studies of alveolar macrophage functions and effects of oxidant gases.

Previous Serial No.: NHLI-324

Principal Investigators: Robert Mason, M.D.
Martha Vaughan, M.D.
Thomas P. Stossel, M.D.

Other Investigators: Willie Thompson

Collaborating Units: Gary Huber, M.D.,
Channing Laboratory, Boston City Hospital.

Project Description:

Objectives:
To investigate (1) synthesis of dipalmitoyl lecithin (DPL), the major component of surfactant in lung and alveolar macrophages, (2) properties of phagocytic vesicles isolated from guinea pig polymorphonuclear leukocytes and rabbit alveolar macrophages, and (3) the effect of oxidant gases on alveolar macrophage function.

Methods:

(1) Disaturated lecithins were isolated by thin-layer chromatography after formation of mercuric acid adducts of purified lecithins. The isolated disaturated lecithin formed from radioactive palmitate was further characterized by degradation with phospholipase A.

(2) Phagocytic vesicles were isolated by sucrose density centrifugation from homogenates of cells which had ingested a mineral oil emulsion. Vesicles were analyzed for content of protein, phospholipid phosphorus, mineral oil, hydrolytic enzymes, and succinic dehydrogenase. Individual phospholipids were separated by two-dimensional thin-layer chromatography. The fatty acid composition of the lecithin and phosphatidyl ethanolamine fractions was determined

by gas-liquid chromatography of the methyl esters of the fatty acids derived from these phospholipids.

(3) Dr. Gary Huber, in the Channing Laboratory, examined alveolar macrophage ultrastructure and bacterial clearance in mice after exposure to 100% oxygen in different treatment regimens. We are determining the protein and phospholipid phosphorus content of saline pulmonary lavage fluids from these animals.

Major Findings:

(1) Last year's finding of synthesis of DPL by rabbit alveolar macrophages in vitro was extended to in vivo studies. The palmitate incorporated into disaturated lecithin, isolated from alveolar macrophages, pulmonary lavage fluid and whole lung, was nearly equally distributed in the alpha (I) and beta (II) positions. The similarity of the distribution of the incorporated palmitate implies a similarity of synthetic pathways. No selective effect of thyroxine or corticosteroids on the content or synthesis of DPL in whole lung was demonstrable. (Last year it was found that steroid treatment caused an increase in the protein, phospholipid and macrophage content of pulmonary lavage fluid.) No specific alteration in rate of synthesis of DPL, studied with labeled palmitate, glycerol or lysolecithin, was found in alveolar macrophages or guinea pig PMN during phagocytosis.

(2) The phospholipid composition of phagocytic vesicles from PMN is strikingly similar to that of the remainder of the cell, whereas the vesicles from alveolar macrophages, when compared with the whole cells, have greater percentage of sphingomyelin and polyglycerol phosphatide (tentatively identified as phosphatidyl glycerol) and a lesser percentage of phosphatidyl ethanolamine and lecithin. The lecithins were subfractionated into disaturated and unsaturated species, and in both cell types there was an increased percentage of disaturated lecithin in the phagocytic vesicle. Fatty acid analyses are incomplete.

(3) Intermittent exposure of mice to 100% oxygen induced tolerance to the ultrastructural damage and impairment of bacterial inactivation usually produced by prolonged exposure. Analyses of the saline pulmonary lavage fluid are in progress.

Significance to Heart and Lung Research:

(1) DPL is the primary physiologic constituent of pulmonary surfactant, which is important for maintainence of the ventilatory function of the lung. Studies of the metabolism of DPL by alveolar macrophages and lung slices may have direct clinical application.

(2) Since alveolar macrophages are in the first line of defense against inhaled toxic materials, their phagocytic function is highly important to the lung. Characterization of their phagocytic vesicles may aid in understanding the phagocytic process.

(3) Since oxidant exposure is a constant factor for alveolar macrophages and 100% oxygen is necessary for certain desperate clinical situations, understanding and modifying the toxicity of 100% oxygen may be of immense benefit in caring for patients.

Proposed Course:

These projects will be completed by July 1971, at which time one of the principal investigators (RM) is leaving NIH.

Publications:

Huber, G., Mason, R., Gardner, D., and Coffin, D.: Alterations in the Lung following the administration of ozone. Archives of Internal Medicine, in press, 1971.

Serial No. NHLI-293
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

NIH - PHS
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Metabolism of Phagocytic Cells.

Previous Serial No.: NHLI-326

Principal Investigators: Thomas P. Stossel, M.D.
Martha Vaughan, M.D.

Other Investigators: Robert J. Mason, M.D.

Collaborating Units: Thomas D. Pollard, M.D., Section on Ultrastructure, Laboratory of Biochemistry, NHLI
Richard K. Root, M.D., Laboratory of Clinical Investigation, NIAID.

Project Description:

Objectives:
(1) To establish methods for analyzing phagocytosis from a biochemical standpoint, and (2) To use these methods to compare phagocytosis by polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM), and to investigate phagocytosis by peripheral blood leukocytes of patients with putative defects in phagocytic function.

Methods:
Emulsified paraffin oil was used as the substrate for phagocytosis. The buoyant density of these particles facilitated the isolation of pure phagocytic vesicles (PV) by density gradient centrifugation of homogenates. Paraffin oil containing Oil Red O was used to measure the rate of phagocytosis. PMN were obtained from guinea pig peritoneal exudates induced with sodium caseinate. Human leukocytes were collected from peripheral blood sedimented with dextran. Alveolar macrophages were lavaged from the lungs of normal rabbits and of rabbits which had been previously injected with Freund's adjuvant ("stimulated" macrophages). Enzyme assays, unless otherwise specified, were performed by standard methods, as were determinations of chemical components of subcellular fractions and of homogenates.

Major Findings:

(1) Characterization of PV of guinea pig PMN: The phospholipid-protein weight ratio of these structures was 0.47, and the cholesterol-phospholipid molar ratio was 0.69. They did not contain DNA, RNA or glycogen. Electron microscopy revealed trilaminar membrane-bounded vesicles enclosing some

electron-dense material. I^{125}-albumin was retained by the PV during collection. The PV fractions contained acid hydrolases, myeloperoxidase and cyanide-recistant NADH oxidase, and these activities were apparently derived from the granules of the cell as evidenced by a shift of activity from that fraction to the PV. The enzymes in PV exhibited latency to Triton X-100. No changes in enzyme activities of the supernatant fluid fractions were noted. Catalase was entirely in the soluble fractions of homogenates derived from these cells.

(2) Characterization of the PV of human PMN: These PV were essentially the same as PV derived from guinea pig PMN except that one third the amount of PV were obtained per mg of incubated cell protein after comparable lengths of incubation.

(3) Characterization of PV of rabbit AM: Homogenates and PV of AM did not have alkaline phosphatase or peroxidase activities. Half of the homogenate catalase activity was sedimentable and demonstrated a latency pattern to digitoxin identical to that described for liver particles called peroxisomes. AM also had D-amino acid oxidase activity which is associated with peroxisomes, but did not have urate oxidase activity. PV acquired catalase activity with phagocytosis, and greater amounts of this enzyme were found in the supernatant fraction after phagocytosis. Acid hydrolases were shifted from the granules to the PV without alteration in supernatant fraction activities as found in the PMN. "Stimulated" AM had greater activities of acid hydrolases than AM from normal rabbits, and incorporated considerably more enzyme activity into PV.

(4) Kinetics of Degranulation: Rates of enzyme translocation from granules to isolated PV were determined for guinea pig PMN. Enzymes were transferred only while phagocytosis was occurring. During this time, peroxidase, β-glucuronidase, and NADH oxidase had a similar rate of transfer which was distinct from acid phosphatase and alkaline phosphatase. Rabbit AM also degranulated only during the phase of active particle uptake.

(5) Iodination of Protein by guinea pig PMN: PMN incorporate iodide into protein during phagocytosis, presumably by generating hydrogen peroxide in the presence of peroxidase. This phenomenon is presumed to be a bactericidal mechanism. Fractionation of guinea pig PMN incubated with I^{125} revealed that the iodination occurs solely within the PV which provides strong support for the hypothesis that iodination is an antimicrobial concomitant of phagocytosis.

(6) Degranulation of Leukocytes from Patients with Chronic Granulomatous Disease (CGD) and the Chediak-Higashi Syndrome (CHS): Controversy exists whether CGD leukocytes degranulate normally. Three patients with CGD were studied and had normal enzyme specific activities (to protein and Oil Red O) in PV (hence normal degranulation). Two patients with CHS, however, had low specific activities of β-glucuronidase and peroxidase in PV.

(7) Studies of Oxidases in PMN: Two fluorimetric assays were developed for measuring cyanide-resistant pyridine nucleotide oxidase activities (disappearance of NADH and generation of NAD). These enzymes have been implicated in hydrogen peroxide generation by PMN during phagocytosis. Homogenates and subcellular fractions were considerably more active with respect to NADH than NADPH. No NADPH oxidase activity was detectable in human or guinea pig PMN PV. Attempts to assay H_2O_2 generation in the presence of NADH or NADPH were unsuccessful. Unusual substrate activity behavior was found for NADH oxidase, and the enzyme was unstable. No enzyme activation was detected in homogenates or subcellular fractions prepared from phagocytosing cells. A "low Km" (0.6 mM) NADH oxidase form was associated with isolated human and guinea pig PV as opposed to other fractions and whole homogenates which had two apparent Km forms of the enzyme. NADH oxidase activity was not different from normal (total or specific activities) in subcellular fractions of CGD leukocytes (which clearly failed to produce H_2O_2 during phagocytosis).

(8) Rate of Phagocytosis: Paraffin oil containing Oil Red O emulsified with fraction V from bovine serum and a standard suspension of guinea pig PMN was used to establish the time course, optimal medium, the relationship between phagocytic rate and the metabolic concomitants of phagocytosis, and the effects of drugs, hormones and inhibitors on phagocytosis. The rate assay was satisfactory principally because uningested particles could be easily washed from cells and did not adhere to them. The phagocytic rate was strongly dependent on Mg^{++} concentration in the medium, and the metabolic alterations were a function of initial phagocytic rate. The sensitivity and reliability of the assay allowed demonstration of inhibitory actions by N-ethyl maleimide, colchicine, hydrocortisone, theophylline, and dibutyryl cyclic AMP. These agents had previously been thought to cause metabolic alterations without affecting the rate of phagocytosis when added to cells in dosages employed in this study. Insulin had a slight stimulatory effect on the rate of phagocytosis. The question of recognition, i.e., the chemical nature of the particle surface which signals the cell to ingest it, was approached by using different substances to prepare paraffin oil emulsions. Preliminary results suggest a complex interaction between the surface of the particle and divalent cations in the medium which produce a favorable net surface charge.

(9) An in vitro Model for "Bacterial" Phagocytosis: Paraffin oil emulsified with E. coli lipopolysaccharide endotoxin requires opsonization by serum for phagocytosis by guinea pig PMN. Modification of the serum by heat, zymosan treatment, and omission of "natural" antibody, alter the rate of phagocytosis in the same manner that has been described for phagocytosis of E. coli organisms. This approach may establish a model for precise quantitation of immunological events related to phagocytosis.

Significance to Heart and Lung Research:

Phagocytosis is a fundamental cellular function relevant to host defense and the pathogenesis of inflammatory and degenerative processes occurring in all parts of the organism.

Proposed Course:
These projects will all terminate by June 30, 1971.

Publications:

Stossel, T.P., Murad, F., Mason, R.J., and Vaughan, M.: Regulation of Glycogen Metabolism in Polymorphonuclear Leukocytes. J. Biol. Chem., 245 (22): 6228-6234, 1970.

Stossel, T.P., Pollard, T.D., Mason, R.J., and Vaughan, M.: Isolation and Properties of Phagocytic Vesicles from Polymorphonuclear Leukocytes. J. Clin. Invest., in press, 1971.

Serial No. NHLI-294
1. Molecular Disease Branch
2. Section on Metabolism
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Studies on the Lipolytic Enzymes of Adipose Tissue.

Previous Serial No.: NHLI-327

Principal Investigators: Su-Chen Tsai, Ph.D.
Martha Vaughan, M.D.

Other Investigators: Ferol Lieberman, M.S.

Project Description:

Objectives:
(1) To develop methods for the purification of the lipolytic enzymes of rat adipose tissue, with special attention to the hormone-sensitive lipase, and (2) To investigate the mechanisms of activation and inactivation of the hormone-sensitive lipase, specifically the role of protein kinases.

Methods:
Fractionation of adipose tissue homogenates by standard methods in an attempt to separate the hormone-sensitive lipase from other lipases and esterases, and to separate the enzymes that activate and inactivate it. Assay of lipase activity using ^{3}H-oleic acid labeled triolein as substrate; assay of esterase activity using ^{3}H-glycerol labeled monoolein.

Major Findings:

(1) Inactivation of Lipase: Lipase in the 100,000 g supernatant fluid from water or 0.25 M sucrose homogenates was inactivated by incubation with 2-4 mM ATP-$MgCl_2$. Maximum inactivation (usually 70-75%) was reached after 20-30 minutes of incubation. Inactivation was completely prevented by 0.25 mM EDTA. The effect of EDTA was not reversed by addition of Mg^{++}, Ca^{++}, Zn^{++}, Co^{++}, Fe^{++}, Fe^{+++} or Ba^{++} ions. Phosphocreatine, but not GTP, ADP, AMP or Na-pyrophosphate, could be substituted for ATP. Ca^{++}, Mn^{++}, Fe^{++} or Ba^{++} could not be substituted for Mg^{++} ion. The lipase remained inactivated after passage through Sephadex G-25 or precipitation.

We had previously found that most of the lipase activity of the 100,000 g supernatant could be precipitated at pH 5.2 or by ammonium sulfate (15-40% saturation). Incubation of these fractions with ATP-Mg^{++} did not lead to inactivation unless a small amount of whole supernatant fluid or supernatant from pH 5 precipitation was added. When whole supernatant fluid was eluted through Sephadex G-25, the protein peak contained lipase activity which was

not activated by ATP-Mg. Addition of material from fractions eluted immediately after the protein peak restored ATP-Mg inactivation. It was effective also with the ammonium sulfate precipitated lipase. Active fractions lyophilized after addition of a small amount of albumin were stable as long as stored dry at -80°. Activity was lost with repeated freezing and thawing, with heating at 65° for 4 minutes, or with dialysis. Using this fraction and the ammonium sulfate precipitated lipase, we have investigated the effects of varying concentrations of the components of the system on the rate and extent of lipase inactivation.

(2) Activation of Lipase: We reported last year that lipase in the ammonium sulfate fraction could be activated (usually 25-40%) by incubation with ATP, $MgCl_2$ and cyclic AMP. It seemed probable that a cyclic AMP stimulated protein kinase was involved in this process, but we were unable to demonstrate enhanced activation with addition of partially purified protein kinase from adipose tissue. We have now found that when the lipase (ammonium sulfate fraction) is sufficiently diluted, activation by ATP, Mg^{++} and cAMP is dependent on the addition of protein kinase (partially purified from skeletal muscle).

After inactivation with ATP and Mg^{++} as described above, partial reactivation of the lipase can be achieved by incubation with the protein kinase plus ATP, Mg and cyclic AMP.

Significance to Heart Research:

Availability of plasma FFA, an important energy substrate for the heart, is regulated via the hormone-sensitive lipase activity of adipose tissue. Purification of this enzyme is essential before the mechanisms through which its activity is regulated can be elucidated.

Proposed Course:

We plan to continue investigation of the activation and inactivation of the lipolytic enzymes, especially the hormone-sensitive lipase, by protein kinase.

Publications:

Tsai, S-C., Belfrage, P. and Vaughan, M.: Activation of hormone-sensitive lipase in extracts of adipose tissue. J. Lipid Research, 11: 466-472, 1970.

Serial No. NHLI-295
1. Molecular Disease Branch
2. Human Biochemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: The Mechanism of Hemoglobin Biosynthesis in Rabbit Reticulocyte Cell-Free Systems

Previous Serial No.: NHI-315

Principal Investigators: W. French Anderson, M.D.
David A. Shafritz, M.D.
Philip M. Prichard, Ph.D.
Ronald G. Crystal, M.D.
David G. Laycock, Ph.D.
Alita Rosenfeld, Ph.D.

Other Investigators: Dante Picciano, M.S.

Cooperating Units: None

Project Description:

Objectives: The mechanism and regulation of mammalian protein synthesis is being investigated by utilizing hemoglobin biosynthesis as a model system. The approach has been to develop an active cell-free protein synthesizing system from rabbit reticulocytes. By fractionating this cell-free system, it has been possible to identify several of the cellular components involved in the process of initiation of hemoglobin synthesis.

Methods: Reticulocytes from phenylhydrazine-treated rabbits are lysed and the known components of the protein synthesizing machinery are isolated and purified. Protein factors and tRNA species which are required for hemoglobin biosynthesis are examined both in terms of their direct role in protein synthesis, and in terms of their characteristics in isolated enzymatic reactions.

Major Findings: 1) Three protein initiation factors, M_1, M_2 and M_3, have been partially purified and characterized. M_1 is a binding factor; M_2 is a factor which specifically recognizes the mammalian initiator tRNA; and M_3 is a factor which is specific for

natural mRNA templates.

2) The initiator tRNA for hemoglobin biosynthesis has been identified as Met-$tRNA_F$. Not only is Met-$tRNA_F$ specifically bound to reticulocyte ribosomes by the initiation factors M_1, and M_2, but also Met-$tRNA_F$, under the direction of the initiation factors, donates the first amino acid of the nascent hemoglobin chain.

3) Examination of the energy requirements of the process of initiation indicates that at least one GTP hydrolysis event occurs during initiation after the binding of the initiator tRNA to the 40S subunit. This conclusion follows from the observation that GDPCP will substitute for GTP in Met-$tRNA_F$ binding but not in Met_F-puromycin formation; furthermore, fusidic acid, an antibiotic that blocks GTP hydrolysis, has no effect on the former reaction, but totally inhibits the latter.

4) M_2 has been separated into two components on the basis of size, M_{2A} and M_{2B}, both of which are required for Met-$tRNA_F$ binding and Met_F-puromycin formation. M_{2A} has 40S dependent ^{32}P-GTP hydrolysis activity.

Significance to Biomedical Research and Institute Program: An understanding of the mechanism and regulation of mammalian protein synthesis is important to the understanding of overall normal cell function.

Proposed Course of Project: Purification of the mammalian initiation factors followed by studies to determine their mechanism of action; use of the initiation factors and the initiator tRNA in order to obtain a better understanding of the initiation process in mammalian cells; sequencing of the initiation region of hemoglobin mRNA.

Honors and Awards: None

Publications:

1. Shafritz, D.A. and Anderson, W.F.: Factor dependent binding of methionyl-tRNA's to reticulocyte ribosomes. Nature 227: 918-920, 1970.

2. Shafritz, D.A. and Anderson, W.F.: Isolation and partial characterization of reticulocyte factors, M_1, and M_2. J. Biol. Chem. 245, 5553-5559, 1970.

3. Shafritz, D.A., Laycock, D.G., and Anderson, W.F.: Puromycin-peptide bond formation with reticulocyte initiation factors M_1 and M_2. Proc. Nat. Acad. Sci. 68, 496-499, 1971.

4. Anderson, W.F. and Shafritz, D.A.: Met-$tRNA_F$: The initiator tRNA for hemoglobin biosynthesis. Cancer Research, In press.

5. Gilbert, J.M. and Anderson, W.F.: tRNA-dependent cell-free hemoglobin synthesis. Methods in Enzymology. Vol. XII, Part D, Eds. L. Grossman and K. Moldave, Academic Press, Inc., New York, In press.

Serial No. NHLI-296
1. Molecular Disease Branch
2. Human Biochemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Evolutionary Homology of Components of the Protein-Synthesizing Machinery

Previous Serial No.: NHI 316

Principal Investigators: W. French Anderson, M.D.
David A. Shafritz, M.D.
Dante J. Picciano, M.S.

Other Investigators: None

Cooperating Units: None

Project Description:

Objectives: The extent of evolutionary homology of the components of the cell's protein-synthesizing machinery in organisms throughout the phylogenetic tree is being examined.

Methods: Cell components are isolated from organisms ranging from E.coli to human. Interchangeability of components between species is tested in cell-free assays.

Major Findings: 1) E.coli initiation factor F_2 appears to be partially interchangeable with mammalian initiation factor M_1.

2) Initiation factors from rabbit liver are being compared with the reticulocyte initiation factors M_1, M_2, and M_3. The liver 0.5 M KCl ribosomal wash fraction appears to contain enzyme activities similar to the reticulocyte M_1 and M_2.

Significance to Biomedical Research and Institute Program: The fact that at least parts of the cell's genetic apparatus appear to have remained essentially unchanged during the evolutionary development from bacteria to man implies that many conclusions concerning mechanism of cell function drawn from bacteria might be applicable to human tissues. By demonstrating the inter-

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changeability of a component it is possible that genetic apparatus from lower organisms might someday be used therapeutically in man.

Proposed Course of Project: Initiation factors from human tissues as well as from organisms between bacteria and mammals are being examined. The interchangeability of the initiator tRNA Met-$tRNA_F$ is being tested between species.

Honors and Awards: None

Publications: None

Serial No. NHLI-297(c)
1. Molecular Disease Branch
2. Human Biochemistry
3. Bethesda, Maryland

PHS-NHLI
Individual Project Report
July 1, 1970 through June 30, 1971

Project Title: Regulation of Hemoglobin Chain Synthesis in Beta Thalassemia

Previous Serial No.: NHI-317 (c)

Principal Investigators: W. French Anderson, M.D.
Arthur W. Nienhuis, M.D.
Abigail Thornton, M.S.

Other Investigators: David G. Laycock, Ph.D.

Cooperating Units: Robert I. Levy, M.D., NHLI: MDB, Section on Lipoprotein

Project Description:

Objectives: Beta thalassemia, also known as Cooley's anemia, is an hereditary disease characterized by severe anemia and transmitted as a typical Mendelian recessive. Anemia is a consequence of a low level of β chains of hemoglobin produced by the patient's red blood cells. No amino acid change has been found in the hemoglobin β-chains of thalassemic patients. It is generally assumed, therefore, that the molecular abnormality resides in the regulation of β-chain synthesis. Our laboratory is attempting to determine the normal mechanisms for regulation of hemoglobin chain synthesis and to determine the nature of the defect in β-thalassemic cells which allows normal α-chain, but limited or absent β-chain, production.

Methods: Blood is obtained both from patients with beta-thalassemia and from patients displaying a high reticulocyte count secondary to another cause (for example, autoimmune hemolytic anemia, sickle cell anemia, etc.) Cellular components involved in hemoglobin synthesis are isolated and utilized in a cell-free hemoglobin synthesizing system. By substituting each component in turn from the thalassemic cells into the non-thalassemic cell-free system, it should be possible to test each component of the thalassemic system to determine if it is normal or abnormal.

Major Findings: 1) A highly active cell-free globin synthesizing system has been developed using components derived from human and/or rabbit reticulocytes. When using components from non-thalassemic human reticulocytes an α-β globin chain ratio of 1 is obtained; when using components from thalassemic reticulocytes the α/β ratio is 8, similar to the abnormality in intact thalassemic cells.

2) By examining each component separately in a fractionated cell-free system, it can be demonstrated that the ribosomes, initiation factors, supernatant proteins, and tRNA in thalassemic cells are normal.

3) When the messenger RNA component of thalassemic cells is tested in the non-thalassemic system, the abnormal α/β chain ratio can be reproduced. Thus, the molecular defect in β-thalassemia appears to reside in the β-chain mRNA itself, either as a reduction in the absolute amount of mRNA or as an alteration in its nucleotide sequence.

Significance to Biomedical Research and Institute Program: β-thalassemia is a severe hereditary disease which affects a sizeable number of individuals in the Mediterranean and Asiatic countries. A method for treating this disease, which will reduce or eliminate the frequent blood transfusions required, is needed. In addition, the techniques utilized to learn how the rate of hemoglobin synthesis is regulated can then be applied to studying other diseases where the defect is also in the faulty regulation of a gene product.

Proposed Course of Project: Studies are being conducted to determine if the defect in the messenger RNA of β-thalassemic cells is due to an absolute decrease in the amount of β-chain mRNA or if the defect is in the sequence of the mRNA. If the former situation is found, then regulatory processes involved in the transcription of globin mRNA from bone marrow DNA will be examined.

Honors and Awards: None

Publications:

1. Gilbert, J.M., Thornton, A.G., Nienhuis, A.W. and Anderson, W.F.: Cell-free hemoglobin synthesis in beta-thalassemia. Proc. Natl. Acad. Sci. 67, 1854-1861, 1970.

2. Nienhuis, A.W., Laycock, D.G., and Anderson, W.F.: Translation of rabbit hemoglobin mRNA by thalassemic and non-thalassemic ribosomes. Nature, 1971, In press.

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